THE AMERICAN
JOURNAL OF PHYSIOLOGY

VOL. 138 FEBRUARY 1, 1943 No. 3

THE RÔLE OF PRESSORECEPTORS IN THE REGULATION OF BLOOD PRESSURE IN THE RABBIT

THELMA H. SIMISTER AND RUTH E. CONKLIN

From the Department of Physiology, Vassar College

Received for publication August 24, 1942

Recent work on regulatory mechanisms for the circulation have shown that important sources of afferent impulses are located in the carotid sinus, the arch of the aorta and the Pacinian corpuscles of the mesentery. It was confirmed by Heymans *et al.* in 1936 that, in the absence of the sensitive zones in the carotid sinus and aortic arch, changes in general blood pressure still cause compensatory reactions in vascular tone. They suggested that the clue to these reactions might be found in the mesenteric area. Gammon and Bronk (1935) had already noted that the Pacinian corpuscles of the mesenteric area signal the degree of distention of the mesenteric vessels by afferent impulses sent along the splanchnic nerves. Since no observations had been made on the rabbit on the rôle of the splanchnic area in connection with other reflexogenic zones, it was decided to study the effects of elimination of these sources of afferent impulses during tipping experiments in the foot-down position (hind feet down, head up), where compensation for the action of gravity might be expected.

METHOD. The rabbits were anesthetized with urethane, with the addition of some ether during the splanchnic isolation. Blood pressure was recorded with a modified Hürthle manometer from a cannula in the left carotid artery. The average blood pressure was determined by measuring a large number of systolic and diastolic pressures. This method was used in preference to the more laborious one of calculating mean pressures by the planimeter, since a comparison of the two methods showed them to be closely in accord. This had been observed by Edholm also in 1940. In five experiments records of respiration were included, using a pneumograph and tambour.

A special animal board was used for the tipping, arranged so that the angle of rotation was just caudal to the rabbit's heart. Animals were tipped for 20 seconds, in the earlier experiments to 30°, 45° and 60°. Later, as the rabbit's ability to compensate became more evident, the lower degrees of tipping were omitted and only 60° and 75° were used. Only the experiments at 60° and 75° are reported here. To prevent sliding in the upright position the rabbit's hind feet were braced and lightly tied against a stop at the lower end of the board.

Two semi-cylindrical, adjustable metal uprights were used under the axillae. The rubber tubing connected directly with the carotid cannula was encased in a metal collar clamped to the animal board, and held firmly in place by sewing it to the rabbit's skin.

The order in which the nerves were cut was varied, starting sometimes with the vagi and aortic depressors and sometimes with the splanchnics. The vagi in the rabbit contain some depressor fibers, so it was necessary to cut them as well as the separate aortic nerves. The use of the left carotid artery for blood pressure registration eliminated the left carotid sinus. The right carotid sinus was eliminated temporarily during a tipping experiment by clamping the right carotid artery. Clamping had already been compared with denervation (Conklin and Dewey, 1941), as to its effect on the reflexes under consideration here, and had been found to give identical results. The splanchnic nerves were isolated by the extra-peritoneal approach, in all but two cases. Ligatures were laid under them and they were eventually severed by a sharp pull on the ligatures, without changing the position of the animal. At autopsy in each rabbit there was careful verification of all nerves severed. This was especially necessary in the case of the splanchnics, where the number of strands, often obscured by fat, varies from one to five on a single side, and where two sides are seldom alike. No experiment was included in the results where any splanchnic twig was left unsevered.

Results. The responses of normal animals were, in the great majority of cases, as follows: the blood pressure dropped rapidly with the tipping, but showed a marked compensatory rise while the animal was still tipped; a few animals, notably those with low blood pressure to begin with, did not show a compensatory rise, or even had a slight continuous fall, but in general the response was positive and vigorous. Quite often an intermediate rise occurred at about 10 seconds. The characteristic reaction is shown in figure 1, A. The drop in the blood pressure during the tipping period may be seen, then the compensatory rise and the overshoot at the end, when the animal was returned to the horizontal position. This overshoot was nearly always present and was thought to be evidence of increased stroke volume of the heart due to the hydrostatic effect.

When the vagi and aortic nerves were cut the rabbit made the same compensatory response to tipping (fig. 1, B) except that in some cases the fall of pressure was *less* and the recovery *more* complete, undoubtedly due to the removal of the inhibitory vagus impulses. Respiratory waves are prominent in such records.

When, in addition to cutting the vagi and aortic nerves the right carotid artery was temporarily clamped, so that both carotid sinuses were thrown out of commission, there was an immediate increase in the level of the blood pressure before tipping, a smaller fall when tipped, a better compensation, and often a recovery in the horizontal position to a higher pressure than the initial one (fig. 1, C). So far, the experiments corroborate those of Koch (1935) and Conklin and Dewey (1941).

When, following the elimination of the buffer nerves and the carotid sinuses, the splanchnics were cut, there was, of course, a lowering of general blood pressure, but again a compensatory rise during the tipping period (fig. 1, D). When the splanchnic nerves were cut first, followed by cutting of the buffer nerves

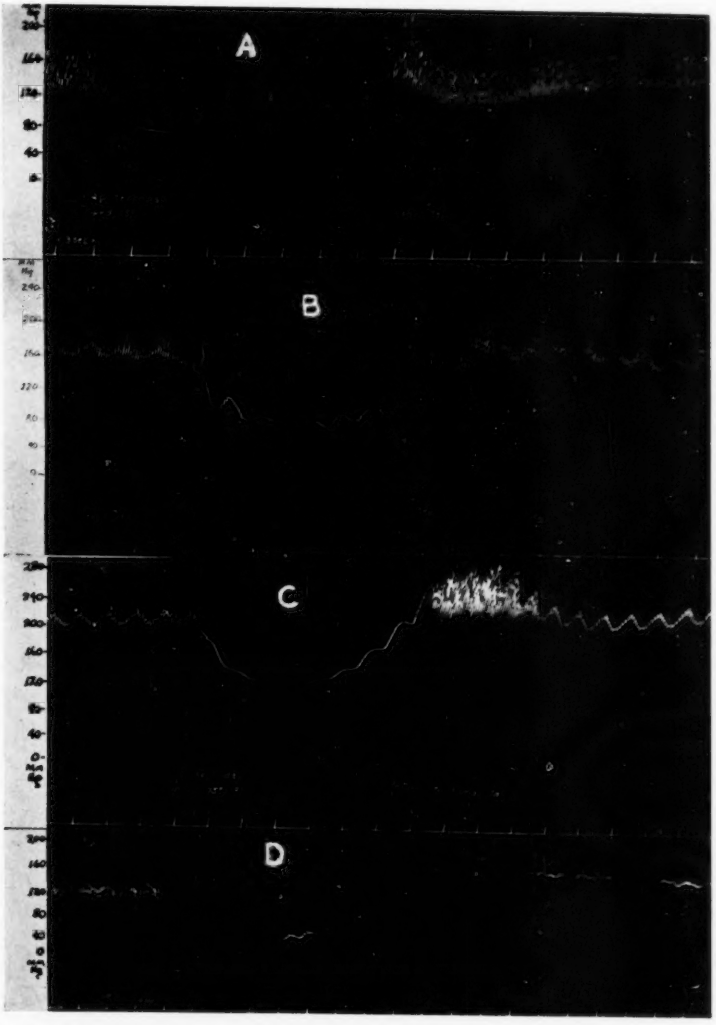


Fig. 1. Blood pressure response to 75° tipping, foot-down position, with progressive elimination of receptor areas. A: normal (cannula in carotid artery). B: vagus and aortic nerves cut. C: vagus and aortic nerves cut, second carotid artery clamped. D: vagus, aortic and splanchnic nerves cut, second carotid artery clamped. Upper line: blood pressure. Middle line: tipping signals. Lowest line: 3-second time signals.

and clamping of the right carotid artery, the effect of splanchnic vasodilatation was rather more marked. There were a few cases where the blood pressure remained the same during the period of tipping. The majority showed a rise, though not a marked one. A rise was also shown in records taken after the splanchnics only had been cut.

Table 1 is a summary of all the experiments at 60° and 75° tipping. It shows the compensatory rise of blood pressure in millimeters Hg during tipping from the time of the lowest pressures reached to the end of 20 seconds, just before the return to the horizontal position. In the experiments with 60° tipping it will be seen that: 1. Elimination of vagus impulses has a favorable effect on compensation. 2. Cutting out both carotid sinuses after vagus section does not disturb compensation, but may even improve it slightly. 3. With elimination of the splanchnics alone, the compensation is lessened, due to splanchnic vasodilatation.

TABLE 1

Mean blood pressure gains during tipping

PROCEDURE (IN ORDER)	60°			75°		
	No. of expts.	Range	Av.	No. of expts.	Range	Av.
		mm. Hg	*mm. Hg*		*mm. Hg*	*mm. Hg*
Normal	8	+8 to +48	+26.5	8	+18 to +62	+34.9
Vagi and aortic nerves cut	8	+26 to +67	+42.0			
Vagi, aortic nerves cut; carotid artery clamped	5	0 to +60	+28.0			
Vagi, aortic nerves cut; carotid artery clamped; splanchnic nerves cut	3	0 to +36	+20.0	2	0 to +28	+14.0
Splanchnic, vagi and aortic nerves cut; carotid artery clamped	8	−2 to +52	+12.0	5	−13 to +35	+10.0
Splanchnic nerves only cut	9	0 to +26	+7.1	6	0 to +27	+13.5

4. Elimination of the buffer nerves, carotid sinuses and splanchnics, whether the splanchnics are cut first or last, still leaves compensatory reactions at work. The experiments at 75°, though not so numerous, show an equivalent response.

In three cases longer tippings at 75° were done on rabbits deprived of buffer nerves, carotid sinuses and splanchnic nerves. A slow rise in pressure occurred in these animals while they remained tipped. One made a gain of 105 mm. in 3 minutes; a second made a gain of 35 mm. in 6 minutes; and a third made a gain of 37 mm. in 9 minutes. These experiments indicate that our 20 second tipping period was too short in some cases to bring out the full capacity of these animals for compensation.

Discussion. The decrease in arterial blood pressure when an individual is tipped to the vertical or near vertical position has been measured in man and several animals. A compensatory rise has been observed in man during the tipping period by many investigators; in cats by Edholm (1940); sometimes in

dogs by Wald, Guernsey and Scott (1937) and Mayerson (1941); and in rabbits by Koch (1935).

It was expected that the rabbit would be even more sensitive than other species to the elimination of the usual sources of afferent impulses. This was found not to be so. It has been shown that the rabbit retains a considerable power of compensation even when all well-known sources of afferent impulses are cut off.

In the cases where respiration was recorded it never failed during the tipping experiments, but was either maintained at the same rate during tipping or at a somewhat reduced rate, if the initial rate had been very high. The shortness of the period during which the animal was tipped and the maintenance of respiration in all cases during this period are indirect evidence against anoxia.

SUMMARY

Brief tipping experiments have been done on 32 rabbits with progressive elimination of sources of afferent impulses causing reflex circulatory compensation. It has been shown that the rabbit is still able to compensate to some extent for the effect of gravity when it is deprived of vagi, aortic nerves, carotid sinuses and splanchnic nerves. Some other reflexogenic sources, responsible for the compensation, must exist.

REFERENCES

CONKLIN, R. E. AND V. C. DEWEY. This Journal **133:** P244, 1941.
EDHOLM, O. G. J. Physiol. **98:** 79, 1940.
GAMMON, G. D. AND D. W. BRONK. This Journal **114:** 77, 1935.
HEYMANS, C., J. J. BOUCKAERT, S. FARBER AND F. Y. HSU. This Journal **117:** 619, 1936.
KOCH, J. Ztschr. f. Biol. **96:** 314, 1935.
MAYERSON, H. S. This Journal **136:** 381, 1942.
WALD, H., M. GUERNSEY AND F. H. SCOTT. Am. Heart J. **14:** 319, 1937.

GLUCONEOGENESIS AND CELLULAR INJURY. A FURTHER INQUIRY INTO THE MECHANISM INVOLVED IN DIABETES ENHANCED BY INFLAMMATION[1,2]

VALY MENKIN

With the assistance of M. A. KADISH and A. A. WARREN

From the Department of Pathology, Harvard University Medical School, Boston, Massachusetts

Received for publication August 3, 1942

It is well known that the course of diabetes is as a rule accentuated by superimposed infection or inflammation. Recent work has indicated that the mechanism responsible for this enhanced condition seems to be primarily referable to an increased degree of proteolysis at the site of inflammation (1). Gluconeogenesis from such protein breakdown products occurs in the inflamed area (1). The excess glucose formed locally in turn diffuses into the circulation, thus giving rise to an excessive hyperglycemia (1). These studies have revealed a higher glucose concentration in the exudate of the inflamed area than in the blood stream, thus supporting strongly other observed facts that the bulk of the sugar is formed at the site of injury rather than originating from the blood.

The earlier literature on the subject has been adequately reviewed elsewhere (1). The present studies represent further data to substantiate the above concept. In brief, glucose apparently seems to be formed in an acutely inflamed area of a non-diabetic dog, but this effect appears to be transitory, for it is soon masked by the developing glycolysis occurring at the site of inflammation. On the other hand, in the diabetic animal the formation of sugar from proteins in an inflamed area is considerably more pronounced and sustained so that it even transcends the elevated level of glycolysis. The result is marked local glucose formation with subsequent diffusion into the circulating blood.

EXPERIMENTAL. The observations were all made on non-diabetic animals and on dogs rendered diabetic by pancreatectomy. The method adopted to withdraw blood samples and exudative material from the pleural cavity of dogs that had previously received an intrapleural injection of about 1.5 cc. of turpentine has been described elsewhere (1). Blood and exudate sugar, total proteins, urea and lactic acid were determined by methods outlined in the earlier study (1). Samples of both exudate and blood were studied at frequent intervals after the introduction of the irritant into the pleural cavity in an endeavor to determine whether a gradient in the concentration of the various substances studied was found to exist between exudate and blood in the earliest stages of the inflammatory reaction. The presence of such a gradient might be of significance in throwing further light on the formation of sugar at the site of an acute inflammation in a diabetic animal.

[1] This study was aided by grants from the Daland Fund of the American Philosophical Society, from the Permanent Science Fund of the Academy of Arts and Sciences, and from the Jane Coffin Childs Memorial Fund for Medical Research.

[2] This article represents paper XXIII of a series entitled "Studies on Inflammation."

RESULTS. I. *Observations on diabetic dogs.* The data on the exudate sugar of diabetic dogs at various stages in the development of the inflammatory reaction are conveniently summarized in table 1 and are shown in chart 1. The levels of blood sugar corresponding to the same intervals are listed alongside. It is clear that in all dogs the exudate sugar is at first at a definitely higher level than the blood sugar. The latter gradually rises reaching conspicuously high concentrations within an interval ranging from several hours to about a day.

TABLE 1

The sugar and urea concentration in exudate and blood of diabetic dogs

DOG NO.	APPROXIMATE DURATION OF INFLAMMATION	EXUDATE SUGAR	BLOOD SUGAR	EXUDATE UREA	BLOOD UREA
	hrs.:mins.	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*
1	0		263.4		36.3
	6:15	500.3	421.1	54.0	50.5
	25:00	537.9	542.4	159.0	132.0
2	0		259.8		11.0
	2:10	351.9	290.0	42.0	33.0
3	0		260.9		17.0
	1:08	309.4	260.7		11.5
	2:18	288.5	258.6		15.5
	4:38	355.0	281.7	19.5	12.0
	7:13	344.8	239.1	23.0	11.5
	25:26	394.8	306.1	41.0	24.5
	48:13	346.8	303.0	37.0	25.5
Reinjected irritant					
Interval after reinjection of irritant	2:22	434.8	377.4	54.0	42.5*
	19:18	566.7	500.0		
4	0		187.8		16.5
	3:36	397.4	264.4	24.0	21.5
	6:08	388.3	283.0	31.0	29.0
	25:03	516.2	421.1	122.5	117.0
Reinjected irritant					
Interval after reinjection of irritant	1:47	625.0	463.0	153.5	142.2
5	0		259.8		27.5
	1:10	342.9	263.2	24.0	23.5
	2:41	396.1	259.1		
	4:30	439.6	291.3	37.5	37.5
	23:19	543.5	357.1	115.5	96.5
Reinjected irritant					
Interval after reinjection of irritant	1:13	571.5	386.6	98.5	87.5
	3:43	535.7	438.6	104.5	93.0
	24:02	471.7	490.1	173.0	161.9
(Dalmatian) 6	0		238.1		21.5
	2:00	381.6	242.9		
	5:20	374.0	201.3	37.0	29.5
	24:18	378.6	234.4	24.0	19.5
	46:55	340.5	220.6	22.5	19.5
Reinjected irritant					
Interval after reinjection of irritant	2:02	333.3	211.3	21.5	17.5
	5:25	242.0	195.5	20.5	13.5
	24:34	218.2	209.1	64.5	53.0

* Blood sample withdrawn a half hour later.

The case of dog 6 is an exception. The blood sugar level in this particular animal, with a superimposed pleural inflammation, fails to be enhanced. It is interesting to note that this animal belongs to the Dalmatian breed known to differ in its purine metabolism, i.e., as regards the excretion of uric acid instead of allantoin. Whether this fact bears any relation to the difference in results obtained is not known. It is also to be recalled in this connection that in a large series of diabetic animals several of them may fail to manifest an enhanced blood sugar with superimposed inflammation (2). In general the findings indicate that the blood sugar tends to approach the level of the exudate sugar about a day

or even at a later period following the introduction of the irritant. Upon reinjection of turpentine in the pleural cavity the concentration of sugar in the exudate is rapidly enhanced to an even higher level. The blood sugar likewise follows such a trend. In brief, the results clearly indicate that with a severe pleural inflammation evidently a gradient due to a lag in the rise in blood sugar is established from the very beginning between the glucose concentration in the exudate and in the blood. This fact suggests that gluconeogenesis occurs at the site of injury. The evidence obtained in control animals, to be presently described, as well as the known inability of glucose to be fixed and thus be concentrated for any appreciable interval in an acutely inflamed area (3) adds further support to the view that the observed gradient can be explained by a process of local gluconeogenesis at the site of inflammation, with gradual diffusion of glucose into the blood stream.

If glucose formation in the inflamed area of a diabetic animal arises from the products of local protein breakdown by deamination, it is reasonable to suppose that some of the constituents of enhanced local protein catabolism might well be recovered in greater concentration at the site of inflammation than in the blood stream. One such product of protein catabolism was therefore studied at length in regard to ascertaining the presence of a gradient between its concentration in the exudate and in the blood stream. This was urea. The data are likewise assembled in table 1. It is clear that in the case of this substance a concentration gradient also exists between exudate and blood. It is, however, considerably less striking than in the case of sugar. This difference may well be referable to the greater diffusibility of urea which would thus tend to be rapidly equalized in concentration in the various body fluids. The diffusion coefficients of urea and of glucose at 15°C. are 0.94 and 0.52 respectively, indicating thus that urea is almost twice as diffusible as glucose. The studies of Bollman, Mann and Magath have indicated that the principal source of urea is the liver (4). This fact, even if correct, is no indication that urea may not likewise be formed, at least to some extent, in an inflamed area. Such studies had not previously been performed. That urea may be formed outside the liver is indicated, for instance, by recent studies showing that the mammary gland is capable of forming urea (5). The work of Krebs and Henseleit has suggested that liver alone is capable of forming urea (6). The mechanism involves the presence of ornithine in this organ. Ammonia and carbon dioxide combine with ornithine to form citrulline which ultimately is converted with the aid of additional ammonia into arginine. The latter in the presence of arginase rapidly forms urea and ornithine. On the other hand, Leuthardt has recently shown that urea can also be formed from glutamine and NH_3. Such synthesis is not catalyzed by ornithine (7). Bach has essentially confirmed and extended the studies of Leuthardt (8). Thus there are two distinct views on the possible formation of urea. It is therefore quite possible that this substance can be formed at the site of acute tissue injury without necessarily resorting directly to the mediation of the contained amino acids of the liver. The data published in the preceding study (1) in addition to the present observations are not incompatible with such a possibility. This

would substantiate further the concept of local gluconeogenesis from the products of enhanced proteolysis at the site of an acute inflammation in a diabetic animal. Further information, however, is still desirable before this important inference can be definitely established as a proven fact.

It is known that glycogen granules have been found in the leukocytes of diabetic patients (9). Is the high glucose concentration found in exudates of diabetic dogs the result of local metabolic disturbances liberating thus sugar from cells injured *in situ* or is this substance merely released by leukocytes which have infiltrated in the inflamed area from the blood stream? There is but little doubt that leukocytes are not essential elements in the process of gluconeogenesis at the site of inflammation in diabetic animals. This can be demonstrated by cytological studies of the exudate at the beginning of the inflammatory reaction. In the first few hours the exudate is found wholly devoid of leukocytes. It contains merely a few scattered desquamated mesothelial cells, doubtless derived in large part from the injured pleura. Yet, a comparison of exudate and blood sugar reveals a conspicuous concentration gradient. The acellular picture, as far as leukocytes are concerned, in the first few hours of inflammation indicates that gluconeogenesis is probably referable to cells injured *in situ* by the presence of the irritant. This would indicate that leukocytes *per se* are not necessary to explain the mechanism of gluconeogenesis involved. It is conceivable, however, that in an inflammatory exudate of about twenty-four hours' duration which at that time contains large quantities of granulocytes, the absolute level of glucose may perhaps be somewhat elevated and thus reinforced by the additional presence of leukocytes.

II. *Observations on non-diabetic dogs.* To control the foregoing observations, similar experiments were repeated on non-diabetic dogs with superimposed pleural inflammation. Samples of exudates were periodically withdrawn by thoracentesis. Blood specimens were obtained by cardiac puncture as described elsewhere (1). The data appear in table 2. An analysis of the measurements reveals in the first few hours of inflammation a concentration of glucose in the exudate that is considerably higher than in the blood stream. This initial heightening in the level of exudate sugar is reflected to some extent in an increase in blood sugar in at least two of the animals. Contrary, however, to the findings in the diabetic animals the enhanced exudate sugar is not sustained, but soon drops to a level which may be even lower than that of the blood sugar (table 2; chart 1). Reinjection of the irritant induces again a transient rise in exudate sugar. These findings differ from the results observed in diabetic animals in so far as in the latter the exudate sugar is maintained at a higher level than the blood sugar throughout the duration of the experiment (cf. chart 1). Furthermore, the apparent continuous production of sugar at the site of inflammation in a diabetic animal is reflected in the circulation where a gradual heightening in blood sugar thereby develops.

Is the transient increase in exudate sugar in non-diabetic dogs referable primarily to an accumulation of this substance from the circulating blood owing to local increased capillary permeability? That this may be in part the state of

affairs is not contended in view of the well known seepage of material from the blood stream into an inflamed area (10). The results in the case of dog 9 (table 2), for instance, substantiate such an interpretation. Furthermore, the inability of glucose to be fixed or concentrated in an area of acute inflammation would reasonably explain the transitory augmentation in exudate sugar of non-diabetic animals as an initial penetration from the circulating blood (3). It is difficult, however, to accept this explanation as the only mechanism involved in view of certain discrepancies. Examination of the data obtained in dogs 7 and 8 reveals

TABLE 2

The sugar and urea concentration in exudate and blood of non-diabetic dogs

DOG NO.	APPROXIMATE DURATION OF INFLAMMATION	EXUDATE SUGAR	BLOOD SUGAR	EXUDATE UREA	BLOOD UREA	DOG NO.	APPROXIMATE DURATION OF INFLAMMATION	EXUDATE SUGAR	BLOOD SUGAR	EXUDATE UREA	BLOOD UREA
	hrs.:mins.	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*		*hrs.:mins.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*
7	0		84.7		29.0	10	0		92.2		13.5
	1:30	189.6	120.3	43.0	37.0		2:05	134.2	102.6	23.0	22.0
	3:30	292.0	180.6	48.0	43.0		4:40	64.5	91.4	17.0	15.0
	25:25	68.6	114.9	30.0	28.0		26:28	97.6	114.3	31.0	23.0
							47:10	97.3	104.5	18.0	14.5
8	0		100.0								
	2:00	248.5	151.0			(Partially	0		99.8		16.5
	6:30	70.6	85.5	67.0	58.5	depancreatized)	1:55	178.6	107.3	30.0	27.5
	27:13	59.7	82.7	37.5	37.0		5:15	119.4	96.3	23.5	23.5
Reinjected irritant						11	22:45	91.1	101.3		
Interval after reinjection of irritant	1:47	288.7	117.6	23.0							
	5:27	60.3	110.2								
9	0		82.3		54.5						
	2:00	125.9	76.9								
	3:40	167.4	95.7	75.0	64.5						
	6:04	78.9	59.5	56.0	47.0						
	24:44	65.7	55.8	30.5	24.5						
	46:36	128.6	81.2	37.0	29.5						
Reinjected irritant											
Interval after reinjection of irritant	1:49	155.7	86.4	36.0	31.5						
	5:52	40.4	76.7	55.5	52.0						

that the initial rise in exudate level is reflected likewise in the blood sugar. If the transient augmentation in exudate sugar in non-diabetic animals is solely referable to an accumulation from the circulation, it is difficult to explain a concomitant tendency for the blood sugar to rise. One would expect a drop, if anything, unless one postulates the entrance of an immediate over compensatory mechanism on the part of the liver to increase the blood sugar level. There is no available evidence for the latter supposition, but nevertheless it is necessary to bear it in mind pending the repetition of these experiments on hepatectomized animals. At any rate on the basis of earlier observations on the penetration of material into an inflamed area (10), the accumulation of sugar from the circula-

tion would certainly be maintained for a considerable interval; and therefore if the liver played a significant compensatory glycogenolytic rôle this might be perhaps expected to be sustained. Yet the rise in blood sugar is only transient. In view of these facts it is therefore quite likely that the initial increase in exudate sugar in non-diabetic animals is also referable, at least in part, to local gluconeogenesis which in turn may be reflected in the blood stream by diffusion of glucose from the site of inflammation.

It is important to explain the transient nature of the rise in exudate and blood sugar in non-diabetic animals in contrast to the sustained effect in depancreatized dogs. In an earlier study it had been shown that the rate of glycolysis in an inflamed area is conspicuous, and in fact that it is considerably more marked than in the circulating blood (11). The gradual production of lactic acid thus adequately explains the mechanism of the developing local acidosis in an area of acute inflammation. It is therefore conceivable that in the inflamed area of a non-diabetic animal, gluconeogenesis at first transcends the initially mild glycolytic process. At a later stage, however, the latter reaction dominates the picture and in turn overshadows the effect of glucose formation. According to this view, gluconeogenesis is thus ultimately masked by glycolysis in the area of acute inflammation of a non-diabetic animal. The following data on two dogs seem to support such an interpretation:

DOG. NO.	DURATION OF INFLAMMATION	EXUDATE SUGAR	EXUDATE LACTIC ACID
	hrs.:mins.	*mgm./100 cc.*	*mgm./100 cc.*
7	3:30	292.0	17.8
	25:25	68.6	35.2
9	1:49 (after reinjection of irritant)	155.7	25.7
	5:52 (after reinjection of irritant)	40.4	95.5

It is clear that with the transient high exudate sugar in the initial phase there is a corresponding low concentration of lactic acid. The reverse order is found in the later stages of the inflammatory reaction. In brief, these facts probably explain satisfactorily, at least, in part, the initially elevated glucose level in the exudate of non-diabetic animals. It is, however, to be remembered that the final picture is doubtless somewhat altered, at least in some animals, by the diffusion from the blood stream of some glucose into the area of acute injury. In the diabetic dogs, on the other hand, there is an exaggerated degree of gluconeogenesis from non-carbohydrate precursors. Even though the process of glycolysis is distinctly more pronounced than in the exudate of non-diabetic animals, the overproduction of sugar dominates the picture inducing thus a sustained effect. This interpretation is fully substantiated by previous observations (1).

A comparison in the lactic acid and sugar levels in exudates of diabetic and non-diabetic animals indicated a rise of only 52 per cent in lactic acid and one of 473.6 per cent in the sugar of diabetic exudates (1). The facts summarized above support further the interpretation of various authors that carbohydrate metabolism in diabetes is merely a quantitative exaggeration of a normal process (12). The sum total of all observations on the trend of the exudate and blood sugar in both diabetic and non-diabetic animals is conveniently represented graphically in chart 1.

TABLE 3

The protein content of exudate and of serum in diabetic and non-diabetic dogs

DOG. NO.	APPROXIMATE DURATION OF INFLAMMATION	TOTAL PROTEIN OF EXUDATE	TOTAL PROTEIN OF SERUM	DOG NO.	APPROXIMATE DURATION OF INFLAMMATION	TOTAL PROTEIN OF EXUDATE	TOTAL PROTEIN OF SERUM
Experimental group (diabetic animals)				Control group (non-diabetic animals)			
	hrs.: mins.	*gm. per 100 cc.*	*gm. per 100 cc.*		*hrs.:mins.*	*gm. per 100 cc.*	*gm. per 100 cc.*
1	6:13	5.3	6.2	7	3:30	9.1	5.3
	25:00	4.6	5.6		25:25	4.9	4.5
5	4:30	4.6	5.5	9	3:40	10.8	6.6
	23:20	4.8	4.7		6:04	7.7	6.6
6	2:00	3.3	5.5		24:44	4.9	7.5
	46:55	3.4	4.9		46:36	4.7	6.0
				10	2:05	8.5	4.3
					4:40	5.3	4.6
					26:28	4.2	4.4
				11	5:15	9.5	6.5
				8*	2:00	4.0	6.9
					6:30	5.5	7.0
					27:13	4.8	5.7

* This animal's protein metabolism seemed to be atypical from the very beginning. For instance, prior to the introduction of the irritant, its blood urea was excessively elevated, namely, 87 mgm. per 100 cc. and its NPN was 58.1 mgm. These high figures were maintained immediately after the introduction of the irritant. Two hours after the onset of the inflammatory reaction the blood urea was 102.5 mgm. per 100 cc. and the exudate urea was 110 mgm. The non-protein nitrogen was 71.9 mgm. in the blood and 65.6 mgm. in the exudate. These unusually elevated base levels in the constituents of protein catabolism may well account for the inconsistent findings in the total protein content encountered in this particular animal.

The foregoing observations suggest that in an area of acute inflammation in a non-diabetic animal there is a certain amount of gluconeogenesis. If the glucose formed locally originates from protein breakdown processes as in the case of diabetic dogs, proteolysis and therefore a somewhat elevated urea level in the exudate might be expected to occur. The data appear in table 2. It is clear that the urea concentration of exudates is a trifle higher than that found in the blood. The difference in levels though definitely small is nevertheless consistent.

III. *Proteolysis in inflamed areas of diabetic and non-diabetic animals.* It is known that the inflammatory reaction is accompanied by proteolysis (13, 1).

Furthermore, previous studies had demonstrated that the local protein breakdown processes in an area of acute injury of a diabetic animal are distinctly enhanced, thus offering a reasonable explanation for the excessive formation of

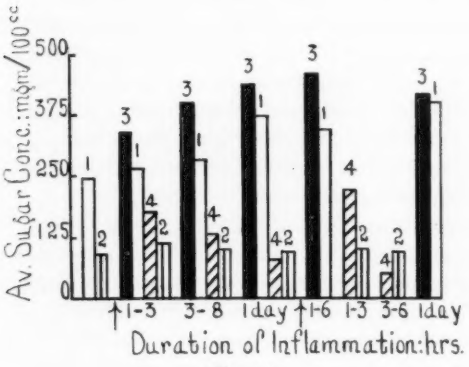


Chart 1

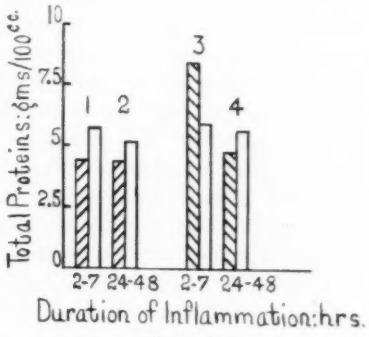


Chart 2

Chart 1. Composite data showing the trend of the exudate and blood sugar levels in diabetic and non-diabetic dogs.

Column 1 represents average blood sugar in diabetic dogs.

Column 2 represents average blood sugar in non-diabetic dogs.

Column 3 represents average exudate sugar in diabetic dogs.

Column 4 represents average exudate sugar in non-diabetic dogs.

The first arrow on the left represents the time of injection of the irritant. The arrow to the right represents the time of reinjection of the irritant. The time indicated after the second arrow on the right refers to the interval which has elapsed following the reinjection of the irritant.

Chart 2. Average protein content of exudate and of serum in diabetic and non-diabetic dogs. Note the intense degree of proteolysis in the initial stage of inflammation in diabetic animals. As a result, the protein content of exudates is lower than that of blood serum. On the contrary, in the non-diabetic animals the process is considerably milder. The effect of increased capillary permeability at the site of inflammation in the earliest stage of the reaction transcends the relatively milder proteolytic process. The consequence is an outpouring of proteins from the circulation into the area of injury, and therefore the protein content of the exudate temporarily rises to an even higher level than that found in the blood serum. Subsequently the extent of local proteolysis overcomes this initial effect and the total protein content of exudates drops to a level below that of the serum proteins.

The protein content of the exudate of diabetic animals is represented in the columns with diagonal lines drawn downward from right to left.

The protein content of the blood serum of diabetic and non-diabetic animals is represented in the blank columns.

The protein content of the exudate of non-diabetic animals is represented in columns with diagonal lines drawn downward from left to right.

Columns labelled 1 and 2 refer to the material studied in diabetic animals.

Columns labelled 3 and 4 refer to the material studied in non-diabetic animals.

sugar from non-carbohydrate precursors (1). A study was undertaken to compare the protein content of exudate and of blood in diabetic and non-diabetic dogs at varying stages of the inflammatory reaction. The observations are conveniently assembled in table 3 and in chart 2.

In the experimental group of depancreatized dogs the total protein level of exudates is distinctly lower than that of blood serum. It is of significance to note that this difference occurs in the earliest stages of the reaction. For instance, an inflammation of two hours' duration reveals a lower protein level in the exudate than the corresponding level in the serum. Such evidence indicates a high rate of proteolysis in the exudate of diabetic dogs. The effect of the increase in capillary permeability which would favor the seepage of plasma proteins from the circulation into the area of injury (14), and thus elevate the level of proteins in the exudate is evidently nullified by the rapid and enhanced rate of local protein breakdown. The result is a lowering in the concentation of total proteins in exudates of diabetic dogs from almost the inception of inflammation.

These findings stand in sharp contrast to the observations recorded on the exudates of non-diabetic animals (table 3 and chart 2). It is clear that in the latter at the beginning of the inflammation the total proteins are considerably elevated. The level is distinctly higher than that in the blood serum. This difference is doubtless referable to an early enhanced capillary permeability favoring the passage of proteins from the blood into the area of acute injury (14). Subsequently, the developing proteolysis in the inflamed area transcends this effect and the protein level of exudates becomes definitely lower than that of the serum. For instance, in dog 9, table 3, the total protein content of the exudate is 10.8 grams per 100 cc. when the inflammation is slightly over three and a half hours' duration in comparison with a concentration of 6.6 in the serum. After an interval of about twenty-four hours proteolysis evidently becomes a conspicuous part of the inflammatory reaction. At that time the total protein content of the exudate measures 4.9 grams as compared with 7.5 grams in the serum. These facts indicate that there is a more pronounced degree of proteolysis in the exudate of a diabetic than in that of a non-diabetic dog. This finding adds further support to the view that the greater local gluconeogenesis in the inflamed area of diabetic animals is associated with enhanced proteolysis.*

Discussion. Soskin regards carbohydrate metabolism as a dynamic balance between blood sugar formation by the liver and its utilization in the tissues (15). According to this investigator the height of the blood sugar level itself regulates the degree of inhibition on the part of the hepatic mechanism. Furthermore, Soskin and Mirsky (16) showed that intravenous administration of diphtheria toxin to a dog alters the normal dextrose tolerance curve. The abnormality was referred to injury to the liver. The blood sugar tends to be elevated apparently due to an augmented hepatic glycogenolysis. Can the present observations on non-diabetic animals be referred primarily to such hepatic involvement? In the initial stage following its injection into the pleural cavity some turpentine is probably absorbed into the circulation. This material may, by reaching the liver, injure this organ and thus induce changes in the blood sugar. The present

* As shown in the earlier study (1) the administration of insulin to diabetic animals with superimposed inflammation represses not only local gluconeogenesis in the injured area but likewise the enhanced proteolysis, indicating thus that the formation of glucose is apparently referable to the proteolytic process.

evidence does not fully preclude such a possibility. However, it is to be noted that preliminary studies on the dextrose tolerance curve in a normal dog and in a dog with pleural inflammation induced by turpentine have failed to reveal any appreciable difference. Hepatectomized preparations to be studied in the future may possibly supply additional information on this point. The observations of the data obtained as shown in tables 1, 2 and 3 definitely suggest the plausibility of a different interpretation. In the first place, essentially throughout the duration of inflammation the exudate sugar in diabetic animals is at a consistently higher level than the blood sugar (table 1, chart 1). This occurs in spite of the fact that this substance is extremely diffusible and as shown by Miller (3) cannot be fixed or concentrated at the site of inflammation. In the second place, whereas the initially elevated blood sugar in non-diabetic dogs may favor the interpretation of an increased formation of glucose by the liver (cf. dog 7, table 2), it is difficult to explain on this basis some of the other results which show a relatively elevated exudate sugar and yet an essentially unaltered level of blood sugar (cf. dogs 9 and 10, table 2). Therefore, in view of these facts, it seems more reasonable to interpret the mechanism involved as primarily one of gluconeogenesis at the site of inflammation. When the amount of glucose formed is conspicuously high, some of the material diffuses readily into the circulation. A concentration gradient is thus established characterized by a higher exudate than blood sugar level. When the process of gluconeogenesis at the site of inflammation is not as marked, one may encounter merely an elevated exudate sugar with, however, no appreciable rise in blood sugar (dogs 9 and 10, table 2). In brief, the available data strongly suggest that basically the same mechanism occurs at the site of injury in the non-diabetic as in the diabetic animals. In the latter the process of gluconeogenesis is merely exaggerated. In the non-diabetic animals glycolysis in the inflamed area (10) eventually supervenes and thus tends to obliterate the effect of the initial gluconeogenesis.[3] In the diabetic animals glycolysis is also enhanced, but not to the same extent as glucose production (1). The result in the depancreatized animal is a marked increase in local gluconeogenesis at the site of inflammation; the glucose gradually diffuses into the circulation. Thus the inflamed area seems to behave as an accessory focus of gluconeogenesis from non-carbohydrate precursors simulating in this respect the function of the liver. This does not mean that the hepatic mechanism may not have some influence in regulating, especially in non-diabetic animals, the transitory, initially exaggerated hyperglycemia which may occur as a result of a superimposed inflammation; but any such effect is presumably secondary to the primary mechanism at the site of acute injury. Finally, accumulation of glucose from the circulation into the acutely inflamed area due to increased capillary permeability doubtless affects somewhat the ultimate level of sugar in the exudate; but the diffusibility of this substance as well as the increase in blood sugar

[3] It is quite conceivable that the various degrees of hyperglycemia and glycosuria or the lowered carbohydrate tolerance accompanying numerous unrelated infectious conditions may perhaps have as a common basis local gluconeogenesis by injured cells with subsequent diffusion of glucose into the circulation (17, 18, 19, 20).

observed in some instances concomitantly with the elevated exudate sugar of non-diabetic animals would preclude the rise in exudate sugar as being due primarily to local accumulation from the blood stream. Diffusibility, increased capillary permeability and therefore penetration from the blood stream into the inflamed area, and local glycolysis are all factors which are to be considered but none of them adequately explains the basic mechanism apparently involved, namely local gluconeogenesis by injured cells.[4]

CONCLUSIONS

An acute inflammation in a depancreatized dog is accompanied by a marked degree of local gluconeogenesis. The surplus glucose formed in the inflamed area from products of local protein breakdown diffuses into the circulation, enhancing thus the existing state of hyperglycemia. The concentration of exudate sugar is at a consistently higher level than the blood sugar from the very beginning of the inflammatory reaction. When the inflammation has progressed for about one day, the concentration of blood sugar tends to approach that of the exudate sugar. The establishment of a concentration gradient between the level of exudate and blood sugar strongly supports the view of a gluconeogenetic process at the site of inflammation.

A similar gradient, though not as marked, is found to exist between the urea concentration of exudate and that of blood. The difference in the magnitude of the glucose and urea concentration gradient seems to be primarily referable to the respective diffusion coefficient of these two substances. The extent of proteolysis in the exudate of a diabetic dog is definitely more marked than in that of a non-diabetic animal (table 3). These facts add further support to previous observations that gluconeogenesis in the inflamed area of a diabetic animal is associated with enhanced local protein catabolism (1). The process of local glucose formation is not primarily referable to the presence of leukocytes but rather to cells in general injured at the site of inflammation.

In a non-diabetic animal the concentration of sugar in exudate is at first higher than that in blood. This effect, however, is transient. This, in turn, is contrary to the findings in diabetic dogs. After the inflammation has progressed from several hours to about a day, the sugar level in exudate of non-diabetic dogs drops to a level usually below that of the blood sugar. This lowering in exudate

[4] It may be of interest to determine whether the amount of sugar capable of being formed in an area of acute pleural inflammation in a diabetic dog can reasonably account for the rise in the hyperglycemia of such animals. An estimate of gluconeogenesis made on the basis of an average dog weighing about 8 kgm. and having an inflamed pleural cavity containing a total protein content in the exudate of about 4 per cent yields approximately 2320 mgm. of glucose per 100 cc., i.e., if all the proteins had been deaminized in the conversion to glucose. Such a quantity of sugar diffusing into the circulation from the site of inflammation would augment the blood sugar by over 330 mgm. per 100 cc. Inasmuch as previous studies indicate an increment of about 216 mgm. of dextrose per 100 cc. of blood in diabetic animals with superimposed pleural inflammation, it is clear that there is an ample marginal reservoir of sugar liberated in the inflamed pleural cavity of a diabetic dog to account for the enhancement in hyperglycemia (1).

sugar concentration is referable to an increase in the local glycolytic reaction which thus overshadows the initial effect of glucose formation at the site of an acute inflammation. If, at the beginning of inflammation in a non-diabetic animal, the degree of local gluconeogenesis is marked, the effect may be reflected in the circulation inducing thus a transient hyperglycemia. The available evidence suggests that the temporary elevation in exudate sugar in the inflamed area of a non-diabetic animal is primarily referable to local gluconeogenesis. The difference in reaction from that in diabetic dogs is quantitative in nature. In the latter local gluconeogenesis is sustained and exaggerated. The effect of abundant glucose production in depancreatized animals cannot be readily obliterated by the slightly elevated local glycolysis. The consequence is constant gluconeogenesis; the glucose in turn diffuses into the circulating blood, thus enhancing the diabetic condition. In brief, injured cells, as manifested by inflammation in both diabetic and non-diabetic animals, are characterized by an increase in their protein catabolic processes and by potentially becoming foci of gluconeogenesis.

REFERENCES

(1) MENKIN, V. This Journal **134:** 517, 1941.
(2) GREENE, J. A. AND A. DAVID. This Journal **133:** P302, 1941.
(3) MILLER, R. G. J. Exper. Med. **67:** 619, 1938.
(4) BOLLMAN, J. L., F. C. MANN AND T. B. MAGATH. This Journal **69:** 371, 1924.
(5) GRAHAM, W. R., JR., O. B. HOUCHIN AND C. W. TURNER. J. Biol. Chem. **120:** 29, 1937.
(6) KREBS, H. A. AND K. HENSELEIT. Hoppe-Seyler's Ztschr. **210:** 33, 1932.
(7) LEUTHARDT, F. Hoppe-Seyler's Ztschr. **252:** 238, 1938.
(8) BACH, S. J. Biochem. J. **33:** 1833, 1939.
(9) WARREN, S. The pathology of diabetes mellitus. Lea and Febiger, Philadelphia, 1938.
(10) MENKIN, V. Dynamics of inflammation. MacMillan Co., New York, 1940.
(11) MENKIN, V. AND C. R. WARNER. Am. J. Path. **13:** 25, 1937.
(12) PETERS, J. P. AND D. D. VAN SLYKE. Quantitative clinical chemistry. v. 1, 1932. Williams & Wilkins Co., Baltimore.
(13) MENKIN, V. J. Exper. Med. **67:** 129, 1938.
(14) MENKIN, V. J. Exper. Med. **52:** 201, 1930.
(15) SOSKIN, S. Endocrinology **26:** 297, 1940.
(16) SOSKIN, S. AND I. A. MIRSKY, This Journal **112:** 649, 1935.
(17) BARTLE, T. D. Med. J. and Record **120:** 207, 1924.
(18) GETTLER, A. O. AND A. V. ST. GEORGE. J. A. M. A. **71:** 2033, 1918.
(19) HOLLINGER, A. Deutsch. Arch. klin. Med. **92:** 217, 1908.
(20) OLMSTED, W. H. AND L. P. GAY. Arch. Int. Med. **29:** 384, 1922.

THE OXYGEN CONSUMPTION OF THE SKIN DURING THE HAIR CYCLE IN THE WHITE RAT

EARL O. BUTCHER

From the Biological Laboratory, Hamilton College, Clinton, N. Y.

Received for publication September 2, 1942

Hair growth in the white rat is cyclic, the follicles being active for about sixteen days and inactive for a similar period (1). This cyclic activity may be demonstrated histologically or it may be observed by removing the hair from the back with a depilatory agent at the end of the growing period (age 20 days) and then watching hair eruption externally about the 35th day of life.

Experiments have been conducted for sometime in learning what factors influence the growing and resting stages of the hair follicle. It has been found that the follicles will remain inactive for long periods if rats are fed only enough feed to prevent a loss in body weight (2). If small amounts of chloral hydrate (10–20 mgm.) are administered subcutaneously daily to well fed animals, the hair buds also remain inactive.

Hair growth can be induced on the backs of underfed rats either by the daily administration of thyroxin (0.2 mgm. subcutaneously for 3 days) (3) or by the daily irritation of the skin on the back with some irritant (xylene, benzoic acid, capsicum, cantharides) (3).

When one analyzes these various results, it appears that hair growth resulted either from an increased passage of food or exciting substances through the capillary wall to the hair follicle, or from increased oxidative processes. Other experiments (4) show that the accelerated hair growth following adrenalectomy is not due to the increased passage of fluid.

It seemed appropriate at this time to determine the oxygen consumption of the skin at various intervals during the hair cycle and to see if there was any evidence that oxidative processes influenced the activity and inactivity of the hair follicle.

METHOD. Hair was removed from the back of the rats with sodium sulphide several days prior to the time of determining the oxygen consumption of the skin so that the depilation would not influence the activity of the skin.

When the determinations were to be made, the rat was killed by a blow on the head and uniform slices of skin (approximately 0.5 mm. in thickness and 1 cm. long) were taken from the dorsum. The oxygen consumption of these slices, usually four, was measured volumetrically in air by means of a Fenn microrespirometer.[1] The slices were shaken in Ringer's-phosphate solution (pH—7.2) at a temperature of 37.5°C. Both the tissue and differential flasks of each respirometer contained 1 cc. of medium, and five drops of N/5 $Ba(OH)_2$ were placed in the small compartment of the tissue flask to absorb the carbon dioxide. Four respirometer tubes were usually run at the same time. Their entire prepa-

[1] I am greatly indebted to Dr. J. A. Dye of the Department of Physiology of Cornell University for the use of microrespirometers and other apparatus.

ration required about twenty minutes, and an interval of twenty minutes was allowed for temperature equilibration before the first reading was taken. After the tests were run for two hours, the tissues were dried to a constant weight at 110°C. Results are expressed in cubic millimeters of oxygen consumption per milligram of dry tissue per hour. The amount of tissue taken usually weighed about 10 mgm. after desiccation.

RESULTS. Determinations of the oxygen consumption of the skin of different rats were made daily during the inactive period (20th to 32nd days) of the hair follicle so that changes could be detected in the oxygen consumption of the skin at the conclusion of the growth of the follicle and prior to the beginning of activity in the hair bud.

Two microrespirometers yielded rather consistent and comparable results for the first hour throughout the experiments. Since they correlated so closely, the data for them are described in detail. Figure 1 shows the results in tube A for the first hour during the various days. The skin from three different rats aver-

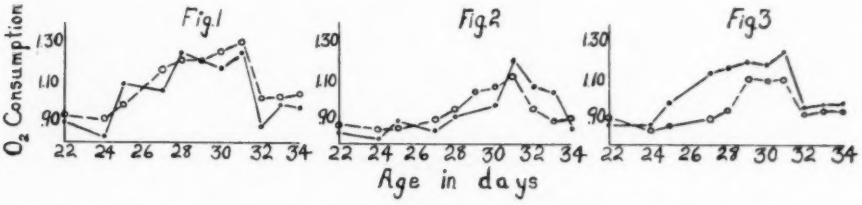


Fig. 1. Oxygen consumption during the first hour. Tube A ·———; tube B o- - -.
Fig. 2. Oxygen consumption during the second hour. Tube A ·———; tube I o- - -.
Fig. 3. Average of all determinations in four tubes. First hour ·———; second hour o- - -.

aged 0.92 cmm. of oxygen per milligram of dry tissue on the 22nd day of life. On the 24th day the skin from four other rats averaged 0.84 cmm. per mgm. This decrease might be due to lowered metabolism following weaning. Oxygen consumption then increased rapidly, reaching 1.26 cmm. per mgm. on the 28th day, or about a 30 per cent increase over that on the 22nd day. The greatest consumption usually occurred on the 31st day. A decrease began after the 31st day. Active growth starts in the hair bud about the 31st day and cells are added rapidly thereafter. The skin is even using more oxygen on the 32nd day than it is on the 30th day. The weight of the skin, however, is being increased by the addition of dead hair cells, and the oxygen consumption per milligram of skin accordingly decreases. Each day's results in figure 1 represents the average of the determinations for at least three rats.

In general the determinations in tube B (fig. 1) follow closely those made in tube A. Again each day's record is the average of the determinations for at least three rats, the same ones which were investigated by the use of tube A. For instance, compare the results in tube A on the 31st day (1.28 cmm. per mgm.) with the average in tube B (1.31 cmm.) for the same three rats.

A study of the determinations in tubes A and I during the second hour (fig. 2) shows a gradual increase of oxygen consumption on the successive days up to the 31st day. Both of these tubes closely correlate, and both show a decline after the 31st day.

Changes in the oxygen consumption of the skin of the individuals of two litters chosen at random may be followed. The results at the various ages represent the average of the determinations in four tubes.

In litter 10 (table 1) the greatest consumption during the first hour is at the age of thirty days. Note the very gradual increase at the various ages. Histological

TABLE 1

Oxygen consumption of the skin of individuals of litter 10

AGE	OXYGEN CONSUMED IN CUBIC MILLIMETERS PER MILLIGRAM OF TISSUE (DRY)		
	First hour	Second hour	Average
days			
24	0.87	0.68	0.78
25	1.00	0.78	0.89
28	1.07	0.93	1.00
30	1.09	0.95	1.02
32	0.90	1.00	0.95
34	0.90	0.85	0.87

TABLE 2

Oxygen consumption of the skin of individuals of litter 11

AGE	OXYGEN CONSUMED IN CUBIC MILLIMETERS PER MILLIGRAM OF TISSUE (DRY)		
	First hour	Second hour	Average
days			
22	0.89	0.93	0.91
24	0.98	1.00	0.99
26	0.94	1.05	1.00
29	1.53	1.35	1.44
31	1.18	1.21	1.19
33	1.02	0.88	0.95

sections of the skin taken on the thirtieth day show no growth whatsoever in the hair buds. Therefore, the oxidative processes increase prior to growth in the hair bud. In litter 11 (table 2) an enormous increase occurs on the 29th day of life. Sections of the skin taken on this day, likewise, show no mitotic activity in the hair bud.

The average of all determinations is shown in figure 3. Each day's record represents the average of at least twelve determinations made in four different tubes. During the first hour the consumption increases rapidly from the 24th day to the 29th day. During the second hour the increase is more gradual. The consumption for the second hour as may be expected is less than that for the first hour.

DISCUSSION. These experiments show that oxidative processes increase prior to activity in the hair follicle. Since the oxidative processes of the skin of underfed animals do not increase and the hair does not grow (unpublished data), it would seem that oxidative processes greatly influence growth, or an increase in the anabolic phase of metabolism is a forwarder of growth. It is entirely possible that the follicle completes growth and oxidative processes awaken it from inactivity.

Processes of maintenance take precedence over those of anabolic growth, and this explains why the hair does not grow upon underfeeding. When underfed animals are treated with either thyroxin or irritants, the oxidative processes or anabolic phase favors growth even with the demand for maintenance. The maintenance demand is not an all out demand or so intense that the animal seriously loses weight. In other words the call of maintenance is not so great but that some substance can be diverted to hair formation.

Thyroxin favors growth in the underfed and well fed animals. From these experiments this would seem to be due to an increase of oxidative processes which the thyroxin causes (5). Others (6) would be inclined to think that the cellular specialization or differentiation in the hair follicle is due to the tyrosine radicle which the thyroxin contains. Administration of 50 mgm. of diiodotyrosine subcutaneously daily for many days in underfed rats failed to induce hair growth. Likewise, 100 mgm. of l-tyrosine daily had no effect. Feeding of l-cystine (40 mgm. daily) also failed to induce growth.

SUMMARY

During the cyclic hair growth in the rat, it has been found that the skin at the conclusion of activity in the hair bulb (age 22 days) is consuming 0.92 cmm. of oxygen per milligram of dry tissue per hour. The oxygen consumption gradually increases and just prior to activity in the hair follicle on the 31st day of life, the skin is consuming 1.31 cmm. per hour. Oxidative processes do not increase in the skin of the underfed animals and the hair doesn't grow. Increase in oxidative processes or the anabolic phase of metabolism just prior to active growth in the hair bud is, therefore, believed to favor the growth of hair in the normal rat.

REFERENCES

(1) BUTCHER, E. O. Anat. Rec. **61:** 5, 1934.
(2) BUTCHER, E. O. J. Nutrition **17:** 151, 1939.
(3) BUTCHER, E. O. This Journal **129:** 553, 1940.
(4) BUTCHER, E. O. AND A. W. GROKOEST. Growth **5:** 175, 1941.
(5) BEST AND TAYLOR. Physiological basis of medical practice. P. 1097, Williams & Wilkins, 1941.
(6) HAMMETT, F. S. Protoplasma **27:** 52, 1936.

THE EFFECT OF AN ENCIRCLING CONDUCTING BAND UPON THE ACTION CURRENTS OF STRIATED MUSCLE

BRUNO KISCH AND MYRON M. SCHWARZSCHILD

From the Departments of Experimental Medicine and Physics, Beth Israel Hospital, New York City

Received for publication September 2, 1942

It has long been recognized that the conductivity and distribution of the material surrounding a muscle may have a profound effect upon the shape of the recorded action potential. That such effects may be fundamental causes for certain alterations of the electrocardiogram has been stressed, particularly by Katz and his collaborators. The effects of immersion in a continuous conducting medium or of a film of conducting material surrounding the muscle form an important part of the now classical study of Craib on the fundamental nature of the electrocardiogram.

These workers have considered the effect of the external medium upon the action current curves for cases in which the medium under consideration surrounds the muscle and has contact with the electrodes.

This report is concerned with the alterations of the action current curves caused by changes in the medium surrounding the muscle which do not directly affect the region of the electrode contact.

The muscles used in these experiments are the retractor capitis muscles of the common turtle. These are long cylindrical muscles inserted in the region of the head with origin at the caudal end of the carapace. The animals were curarized before dissection.

These muscles were supported isometrically in air by means of strings tied to each end. Action currents were recorded with non-polarizable electrodes near each end. The stimulus for the contraction was obtained by the discharge of a small condenser (0.25 microfarad) across the muscle at the end nearer that electrode negativity of which produced an upward deflection. The recordings were made with a cathode ray oscillograph and amplifier, the stimulus being released by a relay suitably arranged in the time sweep circuit of the oscillograph. The amplifier used is condenser coupled with an effective time constant of over 2 seconds.

Tracing A 1 shows a typical record of this kind. The action current wave is a simple biphasic wave. There is no isoelectric interval.

If, now, an encircling band of cotton, about 1.5 cm. wide, soaked in Ringer's solution, a "collar," is placed around the muscle, midway between the electrodes, a profound change is noted in the record. The initial wave is now splintered (tracing A 2). This may be explained simply as a short-circuiting of the advancing doublet while in the region of the conducting collar. Upon removal of the collar the wave returns to the original condition (tracing A 3). The wave of tracing A 1 and that of tracing A 2 are practically identical except for the sharp

dip, as if a portion of the wave were eliminated or suppressed. Similar effects have been observed and similarly explained in nerves by Marmont (1).

In order to demonstrate the correctness of the proposed explanation the following experiment was performed. A single broad collar extending along a segment 6 cm. long was placed about the muscle midway between the electrodes. The resulting action current is shown in tracing B 1. The broad collar was then removed and replaced by two collars 2 cm. wide, placed 2 cm. apart. The result is shown in tracing B 2. These collars were then connected with a salt bridge, i.e., a piece of saline soaked cotton connecting both collars without touching the muscle. The result is shown in tracing B 3. A metal connection

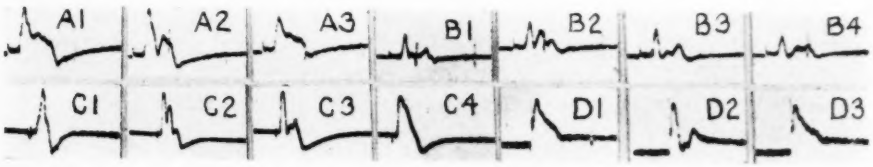


Fig. A 1. Action current of retractor capitis muscle of turtle.

A 2. Same with Ringer's solution collar 1.5 cm. wide midway between electrodes.

A 3. Collar removed.

The time scale of all tracings is 10 cm. per second. Short vertical time marks (0.1 sec.) may be seen on some of the records.

Fig. B 1. Action current with 6 cm. collar.

B 2. Action current with two 2 cm. Ringer's solution collars, with 2 cm. uncovered segment between them.

B 3. Same as B 1 but with saline soaked cotton bridging the gap between the collars, but not touching the muscle.

B 4. Same as B 3 but with metal connection instead of saline bridge.

Fig. C 1. Action current of muscle alone.

C 2. Action current with two Ringer's solution collars.

C 3. Same as C 2 but with collars connected by metal strip not touching muscle.

C 4. Collars removed.

Fig. D 1. Action current of folded muscle with contact of two portions avoided.

D 2. Same as D 1, but with contact.

D 3. Contact again prevented.

of both collars in place of the salt bridge resulted in the wave shown in tracing B 4. In each case after removal of the collar the original wave was again obtained. The effect is thus purely electrical. The collars of Ringer's solution produce no change in the excitation phenomena, but a pronounced change in the electrical manifestations is observed. Another example is shown in tracings C 1–4. Similar collars of tin foil instead of saline produce the same effect. Collars of distilled water or dry collars have no effect.

The question arises as to whether these effects can throw light on the observed predominance of the surface of the heart in determining the form of the electrocardiogram (2, 3). The collar placed about the muscle can obviously have no effect upon any part of the muscle other than its surface. The observed phenomena are another expression of the basic fact that the action currents re-

corded from complete muscles are due to the effects at the muscle surface. This is true no matter what the localization of the actual action potential gradient, whether caused by dipole distributions along the surface of the fibres or dipole distributions across the fibres. The short-circuiting action of the collar may be expected to be the same whichever localization actually prevails in the individual fibre.

Collars such as were used in these experiments may in the future be used as detectors of the passage of excitation for the purpose of velocity measurements. Such a method may, under certain conditions, be much simpler to apply than the usual procedures involving multiple recording.

A single wide collar suppresses a large portion of the initial wave. Thus such a collar results in an action current wave which has two erect components and bears a tantalizing resemblance to the electrocardiogram with its erect R and T wave (tracing B 1). Effects similar to that produced by a wide collar or by two connected collars may be produced by bending the muscle back on itself around an insulating support so that a segment is short circuited by contact. Tracings D 1–3 show the recorded waves for such a case. In tracing D 1 the contact is broken by holding the two portions apart. In tracing D 2 contact is established. In D 3 it is again broken. The R-T appearance is obvious. These results suggest the possibility that the well known twisted nature of parts of cardiac muscle may have some similar influence on the electrocardiogram. In the present unsatisfactory condition of T wave interpretation this possibility may be considered, but we do not believe this to be a complete explanation for various reasons, particularly because of the length of the R-T interval.

SUMMARY

Typical changes are produced in the action potential curves of striated muscle if the muscle be surrounded by a ring or collar of conducting material which does not impinge upon the electrodes. The effect is explained and is related to the predominance of the surface of the heart in the production of the electrocardiogram. The effect may be applied in the measurement of conduction velocity. A modification of the experiment permits the production of action potential curves from striated muscles which closely resemble the R-T of the electrocardiogram.

REFERENCES

(1) Marmont, G. This Journal **130:** 392, 1940.
(2) Kisch, B. Cardiologia **4:** 304, 1940.
(3) Kisch, B., L. H. Nahum and H. E. Hoff. Am. Heart J. **20:** 174, 1940.

REMOVAL OF RED CELLS FROM THE ACTIVE CIRCULATION BY SODIUM PENTOBARBITAL[1]

P. F. HAHN, W. F. BALE AND J. F. BONNER, JR.

From the Departments of Pathology and Radiology of the University of Rochester School of Medicine and Dentistry

Received for publication September 2, 1942

It is commonly observed that the spleen under the influence of sodium pentobarbital (nembutal) becomes engorged and turgid. This is generally considered to be due to the trapping of red cells in the sinusoids. The use of red cells tagged with the[2] radioactive isotope of iron offers a direct means of determining what fraction of the circulating red cells of the body are contained in this organ under these conditions.

Studies of the various forms of shock must inevitably take into consideration the changes which occur in the circulating constituents of the blood as well as the other body fluids. Attention has centered chiefly on variations in plasma volume. Some of the plasma may at times be out of the active circulation (9) and methods employing dilution of dyes may under these conditions yield especially erroneous results. The red cells are *normally* nearly all in active circulation (8) (12) and the measurement of their circulating mass may be carried out by one of several procedures (1) (8). However many studies are made under anesthesia and it is important that the effect of such complicating factors be understood.

Two methods of approach were used in estimating the amount of red cells which might be taken up by the spleen under nembutal, one of these being specific and the other not so. In the first the circulating cells were tagged by injection of donor red cells containing radioactive iron in their constituent hemoglobin. Nembutal anesthesia was induced and the spleen allowed to become engorged. Splenectomy was performed and the organ was subject to wet ashing (7), the iron separated, electroplated, and its radioactivity determined. The total activity in the spleen divided by the concentration of radioactivity in the red cells gave directly the mass of cells in that organ.

The other method consisted in first[3] nembutalising the animal and allowing time for engorgement of the spleen to occur. The actively circulating mass of red cells was then determined by the donor-isotope-cell method (8) (9). The animal was then given a single large dose of epinephrine[4] ($\frac{1}{2}$ ml. of 1:1,000) by vein. After three minutes another sample of blood was drawn and the activity concentration of the circulating red cells determined. Unless circulation in the

[1] We are indebted to the Eli Lilly Company for aid in conducting this work.

[2] We wish to express appreciation to the members of the Radiation Laboratory at Berkeley, California, and particularly to Dr. E. O. Lawrence and M. D. Kamen for the radioactive iron used in these experiments.

[3] Veterinary nembutal, Abbott Laboratories.

[4] Suprarenin, Winthrop Chemical Company.

spleen is as rapid as that of the general circulation, cells for the most part in that organ would not be expected to be tagged under these conditions, and following adrenalin there should have been a further dilution of the circulating red cell radioactivity. This reaction is not specific for the spleen as we shall see below since it is subject to modification by any other part of the systemic circulation which might be affected in a similar manner by nembutal.

Blood from donor dogs was drawn variously into heparin, saturated sodium citrate (1.25 ml. per 100 ml. of blood used), or into isotonic sodium citrate since concomitant studies were being conducted on the survival time of transfused red cells using various anticoagulants. The blood samples of the recipients when taken for sampling were mixed with 5 ml. of isotonic oxalate, 30 ml. of blood usually being taken to allow triplicate hematocrit readings and activity measurements. Cells were centrifuged for 35 minutes at about 2700 r.p.m.

Methods for ashing of blood samples and spleens, separation of iron, and electroplating for determination of activity have been described (5) (7) (9).

Experimental observations. Two dogs were first studied in order to determine the number of red cells contained in the spleen under ether anesthesia. The circulating red cell mass was determined by the isotope-donor-cell method (8) (9). Under ether the splenic blood vessels were then doubly ligated and the organ removed by cutting between the ligatures. After wet ashing the iron of the spleen was separated, electroplated, and the amount of radioactivity measured (5) (9). The total radioactivity of the spleen divided by the concentration of the isotope in the red cells of the circulation then corresponded to the volume of cells in this organ. In the case of dog 41-888, weighing 14 kgm., this amounted to 20 ml. of cells or 3.8 per cent of the total body cells. In the instance of dog 41–807, weighing 13.4 kgm. there were 19 ml. of cells in the spleen corresponding to 2.9 per cent of the total in the body.

Dog 1-G, 10.5 kgm. in weight, was studied to see directly how many red cells might be sequestered by the spleen under the influence of nembutal. A determination of the circulating red cell mass was made by the isotope-donor-cell method. Nembutal was then given by vein in anesthetic dose (27 mgm. per kgm.) and after allowing about seven minutes for the spleen to become engorged, a medial incision was made below the costal margin and the spleen excised as before. The organ was then wet ashed and the iron separated and measurement of the radioactivity done. The total radioactivity in the spleen divided by the concentration of red cell isotope again was a measure of the volume of cells taken up by this organ. In this dog the circulating cell mass was found to be 240 ml. of which 45 ml. or 18 per cent were taken up by the spleen.

Dog 36-196 was treated in exactly the same manner and the circulating cell mass was found to be 354 ml. of which the spleen under the influence of nembutal took up 101 ml. or 29 per cent of the total, table 1. Similarly the cell mass of dog 36-14 was found to be 320 ml. of which 58 ml. or 18 per cent were contained in the spleen.

Another approach was employed in the next few experiments. The dogs were first given the nembutal and after allowing time for the spleen to become en-

gorged with red cells, a determination of the *circulating* red cell mass was done. This latter measurement would presumably exclude the cells sequestered in the spleen provided the circulation rate in this organ were slow compared with the

TABLE 1

Removal of red blood cells from effective circulation by sodium pentobarbital

Direct determination of cells in spleen by splenectomy

EXP.	DOG		RED BLOOD CELL HEMATOCRITS		RED CELL MASS	
			Initial	After inj. donor blood	Total circl.	Per cent in spleen
			per cent	*per cent*	*ml.*	*per cent*
1	1-G	Determination of cell mass tagging all circu-	24.4	21.1	240	18
2	39–196	lating red cells; nembutalised; spleen	43.8	44.8	354	29
3	36–14	ligated and excised, ashed and radioac-	29.0	28.4	320	18
4	2-G	tivity measured	25.7	25.3	268	5

Indirect determination by cell mass dilution following adrenalin

EXP.	DOG		RED BLOOD CELL HEMATOCRITS			CELL MASS		PER CENT SEQUESTERED
			Initial	After inj. donor blood	After adrenalin	Initial	After adrenalin	
			per cent	*per cent*	*per cent*	*ml.*	*ml.*	*per cent*
5	40–15	Nembutalised, sequestering red cells; effec-	50.6	48.9	60.6	645	905	29
6	2-G	tive circulating cell mass determined by	25.7	25.3	29.3	268	274	2
7	36–57	donor-tagged-cell method; injected epi-	49.8	44.2	55.7	560	865	35
8	36–57	nephrine increasing circulating cell mass,	31.6	28.1	39.2	326	368	17
9	36–57	the increment being measured by further	33.0	30.8	41.5	493	585	18
10	38–179	dilution of circulating cell activity	44.2	41.9	58.6	645	1045	37
11	38–179		35.0	33.7	41.3	402	610	34

Splenectomised controls: Determination of cell mass increase after adrenalin

12	39–196	Splenectomised dogs; nembutalised seques-	42.5	42.4	43.8	304	355	14
13	1-G	tering red cells; effective circulating cell	25.5	25.8	28.0	204	242	16
14	2-G	mass determined by donor cell method; injected adrenalin, increasing circulating cell mass, the increment being measured by increased dilution of radioactivity of circulating red cells	23.0	22.8	24.2	240	271	11

rest of the vascular system. Adrenalin was then administered by vein in a single dose of $\frac{1}{2}$ ml. (1:1,000) to cause contraction of the spleen. The resultant outpour of untagged red cells into the circulation resulted in a further dilution of the tagged cells already circulating. Another determination of the concentration of the red cell isotope resulted in a new value for cell mass (total

radioactivity of the injected donor cells divided by the concentration of the isotope in the circulating cells after allowing mixing in the blood stream-cell mass). The difference between the cell mass found before and after the epinephrine was taken to represent the mass of red cells held by the spleen and other tissues under the influence of the nembutal. In table 1 it can be seen that this amounted to 29 per cent of the red cells in dog 40-15; 35 per cent, 17 per cent, and 18 per cent on different occasions in dog 30–57; and 37 per cent and 34 per cent on two occasions in dog 38-179.

In one instance it was attempted to combine these two procedures. Dog 2-G was nembutalised, the spleen brought outside the abdomen, and the cell mass determined as before. It measured 268 ml. Adrenalin was injected and the spleen was seen to shrink down somewhat. No plethysmographic or other volume measurements were made however. Sampling of the blood showed that the cell mass had been increased only about 6 ml. which is well within the experimental error. After allowing a period of fifteen minutes for the epinephrine effect to wear off completely it was hoped that the organ would attain its former size again. Since there was no noticeable increase an additional 180 mgm. of nembutal was administered but made little if any grossly apparent change in spleen size. The organ was excised as before and after ashing and estimation of the radio-iron content, the volume of cells contained was found to be 15 ml. Thus these values accounted for only 2 and 5 per cent respectively of the circulating cell mass, being decidedly lower than those obtained in the other dogs.

Originally planned as controls, the same procedure was applied to the dogs after splenectomy as used in experiments 5 through 11, table 1. Dog 39–196 was given nembutal and the circulating cell mass determined. Epinephrine was injected and another red cell isotope concentration done showing that the cell mass had increased by 14 per cent. The same "control" experiment on dogs 1-G and 2-G showed increases in the cell mass of 16 per cent and 11 per cent respectively.

Where splenectomy was performed under ether the weights of the spleens in three instances were 62, 58 and 77 grams. In three succeeding splenectomies under nembutal the weights of the removed spleens were 211, 235 and 228 grams.

Discussion. In 1936 Essex, Seeley, Higgins and Mann (4) reported that ether anesthesia caused a marked increase in the erythrocyte count, hemoglobin concentration, and the venous hematocrit value in dogs, which effect they ascribed to a profound constriction of the spleen. On the other hand they found that sodium amytal anesthesia caused a drop in the red cell count which they felt was due to the removal of a considerable percentage of the circulating red cells by dilatation of the spleen.

At about the same time Seeley, Essex and Mann (11) reported that sodium amytal alone, or preliminary to ether anesthesia, resulted in a marked delay in the onset of shock produced by intestinal manipulation, as compared to the results with ether alone. Following up these findings Kendrick and Uihlen (10) recently showed that splenectomised animals went into shock more rapidly than the non-splenectomised animals, indicating that the spleen aided the or-

ganism in resisting shock. However with or without spleens shock onset was slower when ether anesthesia was supplemented by nembutal than when ether was used alone.

The experiments in table 1 indicate that under the influence of nembutal there may be a considerable number of red cells sequestered from the active circulation. This is in contrast with what is found in the normal or anemic unanesthetised animal (6). In the first set of experiments (1 through 4), table 1, in which the spleen was excised and the contained red cells measured directly it is apparent that this organ can and does retain a large fraction of the red cells under the influence of this drug. The next set of observations (5 through 11) also indicate that the drug causes the removal of large numbers of cells from the active circulation, but this approach is not specific and fails to show where the cells are pooled. The fact that there may still be about 15 per cent of the cells sequestered in the splenectomised animal under nembutal (12 through 14), table 1, shows that the spleen is not entirely responsible for this reaction, or at least that in the absence of this organ other viscera or the vascular system itself may perform this function. In a larger series it might be seen whether on the average the first and third types of reactions would in their summation give values of the order of the second or less specific group.

In the intact, nembutalised dog there is a marked increase in the venous hematocrit value following the administration of epinephrine as can be seen in experiments 5 through 11, table 1. A similar though less marked increase accompanies the injection of the drug in the unanesthetised dog (2) (3) (6) but under the latter conditions there has not been demonstrated a concomitant increase in the circulating cell mass (6). In the unanesthetised dog following splenectomy the hematocrit response to adrenalin is absent. In the nembutalised dog the reaction is much smaller in magnitude after splenectomy than before but it is not entirely abolished. These findings would indicate that in the normal unanesthetised dog the spleen either contains no appreciable pool of red cells, or that the circulation of blood through the spleen is about as rapid as in the rest of the vascular system. The effect of nembutal would seem to be the sidetracking of a large fraction of the circulating cells, partly by the spleen and partly by other tissues. This nembutal effect is temporarily reversed by the administration of epinephrine.

It is felt that the above experiments may be useful in interpretation of the delayed onset of traumatic shock following the administration of nembutal.

SUMMARY

When the circulating red blood cells of the dog are tagged with other dog cells containing the radioactive isotope of iron, and nembutal anesthesia is induced, removal of the engorged spleen shows that up to 30 per cent of the circulating red cells may be present in this organ as shown by the radioactivity of the contained cells.

When red cells have been sequestered from the circulation by the influence of nembutal, the actively circulating cell mass may be determined by the tagged-

donor-cell technique. Administration of epinephrine by vein results in an increase in the actively circulating cells as shown by dilution of the tagged cells. This increment in circulating cells has been found to be as much as 37 per cent of the total cell mass.

When the latter procedure is applied to splenectomized animals, there is still a marked response to epinephrine, an increment of red cells being added to the circulation. The increased mass of circulating cells under these conditions is about half as great as that obtained in the intact dog.

The spleens removed under nembutal anesthesia were about four times the weight of the organs taken out under ether.

The possible relationship between these findings and the observed delay of onset of traumatic shock following administration of nembutal is suggested.

REFERENCES

(1) Arnold, H. R., E. B. Carrier, H. P. Smith and G. H. Whipple. This Journal **56:** 313, 1921.
(2) Barcroft, J. Lancet **1:** 319, 1925.
(3) Cannon, W. B. and J. J. Izquierdo. This Journal **84:** 545, 1928.
(4) Essex, H. E., S. F. Seeley, G. M. Higgins and F. C. Mann. Proc. Soc. Exper. Biol. and Med. **35:** 154, 1936.
(5) Hahn, P. F. and W. F. Bale. This Journal **136:** 314, 1942.
(6) Hahn, P. F., W. F. Bale and J. F. Bonner, Jr. This Journal, **137,** 717, 1942.
(7) Hahn, P. F., W. F. Bale, E. O. Lawrence and G. H. Whipple. J. Exper. Med. **69:** 739, 1939.
(8) Hahn, P. F., W. M. Balfour, J. F. Ross, W. F. Bale and G. H. Whipple. Science **93:** 87, 1941.
(9) Hahn, P. F., J. F. Ross, W. F. Bale, W. M. Balfour and G. H. Whipple. J. Exper. Med. **75:** 221, 1942.
(10) Kendrick, D. B., Jr. and A. Uihlein. Surgery **12:** 76, 1942.
(11) Seeley, S. F., H. E. Essex and F. C. Mann. Annals Surg. **104:** 332, 1936.
(12) Stead, E. A., Jr. and R. V. Ebert. This Journal **132:** 411, 1941.

QUANTITATIVE MEASUREMENTS OF CEREBRAL BLOOD FLOW IN THE MACACQUE MONKEY[1]

PAUL R. DUMKE AND CARL F. SCHMIDT

From the Laboratory of Pharmacology, University of Pennsylvania

Received for publication September 8, 1942

After reviewing the evidence available in 1936, Wolff (12) made the following statement: "Unfortunately, the amount of blood going to the brain still remains an uncertain quantity." As far as we know nothing has happened subsequently to remedy this situation. In the present paper we report experiments in which cerebral blood flow has been measured quantitatively under conditions which, although not strictly normal, were considerably less abnormal than those existing in perfusions of excised brains—the only circumstances under which comparable measurements have hitherto been made.

The voluminous literature on the physiology and pharmacology of the cerebral circulation has been reviewed recently (1, 4, 12) and need not be discussed here. Reasons for our own interest in these problems, the various methods we have used to study them, and the results obtained, have been presented in a series of publications from this laboratory (4, 5, 6, 7, 8, 9). The latest of these (4) contained an elaboration of the theme of the first (6) in regard to the anatomical and instrumental difficulties involved in quantitative measurements of cerebral blood flow. At that time we hoped, by appropriate modifications, to adapt a thermostromuhr to the purpose. Our misgivings (4, p. 255) regarding the reliability of that instrument for quantitative purposes were proved by our subsequent experience to be well founded, and the recent careful studies of Gregg and his collaborators (2) have amply confirmed them.

In the present experiments we have measured cerebral arterial inflow directly by a method first employed to secure the *in vivo* calibrations of the thermostromuhr already referred to (4, pp. 255 and 263) and by so doing have finally obviated the instrumental difficulties. The anatomical difficulties (4, 6) have been circumvented partly by the use of the monkey (macacus rhesus), in which, as in man, there are only insignificant communications between the intracranial and extracranial parts of the cephalic circulation (1), partly by ligation of the basilar artery, a procedure which not only forces all of the blood entering the brain to pass through the measuring device, but also prevents escape of some of it into extracranial tissues through muscular branches of the vertebral arteries (4, pp. 237 and 254).

METHOD. The measuring device is a refinement of the "simple flowmeter" described by Soskin, Priest and Schutz (10). Several models have been tested but that shown in figure 1 has proved most satisfactory. It is provided with a

[1] This investigation was largely financed through the National Committee for Mental Hygiene from funds granted by the Committee on Research in Dementia Precox founded by the Supreme Council, 33° Scottish Rite, Northern Masonic Jurisdiction, U. S. A.

jacket through which water at a temperature of 38 to 40° is circulated from a thermostatically controlled bath. The blood, rendered incoagulable by heparin, passes through the meter on its way to the brain and the volume of flow is measured by timing the passage of an injected air bubble (about 0.2 cc. in our meters) over a space of known volume (about 6 cc. in most of these experiments). The air bubble is removed by a suitable trap before the blood reënters the arterial circulation and the measurements involve no interference with or alteration in the actual blood flow.

Male monkeys weighing 3 to 6 kilos were used. They were anesthetized with nembutal (about 0.04 gram per kilo intraperitoneally). A tracheal cannula was inserted, blood pressure was recorded by a mercury manometer from a femoral artery with heparin-saline as the anti-coagulant, respiration was registered by a conventional pneumograph-tambour system, and intravenous injections were made through a burette-cannula system connected with a femoral

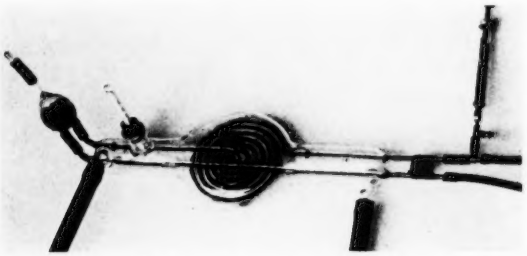


Fig. 1. Bubble flowmeter. A tuberculin syringe is connected through a small Bunsen valve system to a 22 gauge stainless steel tube so that bubbles, each of about 0.2 cc., can be injected into the inflowing stream. The space between the marks on the tube represents about 6.0 cc. The trap for collecting air bubbles is at the left. The large rubber tubes in the foreground connect the water jacket to a thermostatically controlled water bath.

vein. The operation for insertion of the flowmeter involved reflection upward of the larynx and esophagus, exposure of the anterior inferior aspect of the occipital bone, perforation of it by means of a dental burr, opening the dura, and passing a ligature around the basilar artery so that it could subsequently be tied. Silver clips, though easier to apply, turned out to be less dependable than actual ligation. Both common carotids were exposed to a point beyond the bifurcation and both external carotids were tied near their origins. Heparin was given intravenously as an initial 1000 unit (1 cc.) dose followed by 200 units every 15 minutes. A short glass cannula was inserted in each common carotid to collect blood coming from the heart, and these two were connected through a T-tube to the inflow side of the meter. The outflow tube was connected to a similar pair of cannulae inserted more peripherally in the common carotids with their tips pointing cephalad. The location of the needle for injecting the air bubbles is indicated in figure 1. In 6 experiments we attempted to connect a smaller (2 cc.) meter to the cephalic end of the basilar artery, so that flow could be measured in the carotid and basilar systems simultaneously. We succeeded in 3 of these

attempts but in the others the basilar artery was too small or too short for cannulization and we had to be satisfied with measurement of carotid flow with the basilar tied. At the end of each experiment a dye (usually india ink) was injected through the meter and its distribution ascertained by dissection. The only communications with tissues outside the brain were occasional small anastomoses around the basilar ligature, leading to spots of dye in the muscles of the neck, and occasional twigs in the orbits; these channels were so small as to be negligible, we believe. The brain was removed at the end of each experiment and its weight anterior to the basilar ligature was determined.

We have subjected this flowmeter to extensive calibration tests with a perfusion pump, and have also tried it in a number of pilot experiments on dogs and cats. As a result we are satisfied that it is dependable and accurate. There is a tendency toward overestimation of the actual flow, and the magnitude of this error increases both with the volume of the flow and with the viscosity of the fluid, but the deviation is almost imperceptible at flows smaller than 50 cc. per minute and even at the largest flow encountered in our *in vivo* measurements (103 cc. per minute) it would amount to less than 10 per cent. A correction could easily be applied for this but we have not thought it desirable to do so because of the implication of quantitative precision which the operative and other deviations from normal make illusory. The viscosity factor is much smaller than in the venturi meter (11) or rotameter (2). With these, even approximate estimations of flow call for accurate measurements of viscosity, not only for each experiment but for every supposed change in flow. With the bubble flowmeter even the change in viscosity from saline to whole blood has a barely measurable influence at flows lower than 50 cc. per minute; above that level the divergence increases progressively but it is only about 5 per cent at 100 cc. per minute. This independence of viscosity changes is the greatest advantage of the bubble flowmeter over the rotameter.

So far we have successfully employed this method to measure cerebral blood flow in 19 monkeys. The average weight of these animals was 4.2 kilos, the extremes being 3 and 6.2. The average weight of the brain above the level of the basilar ligature was 91 grams and the extremes were 85 and 105.

1. *The volume and range of cerebral blood flow.* The averages of our findings are shown in table 1. The "normal" values are those recorded at the start of each experiment. In many cases blood pressure fell considerably during the final stages of the preparation and in these pressure was restored, by intravenous injection of blood saved from an earlier experiment, approximately to its initial level before the "normal" readings were obtained. The "maximum" and "minimum" figures are derived from the highest and lowest flows recorded in each experiment, terminal states of progressive circulatory failure being excluded. The complete data are omitted in the interests of space conservation. A brief description of their distribution is therefore desirable.

In the 19 experiments in which flow was measured only through the internal carotids the "normal" flows ranged from 27 cc. (0.27 cc. per gram) to 78 cc. (0.81 cc. per gram); the corresponding blood pressure readings were 70 and 140

mm. Hg. The "maximum" flows in 7 instances were measured after intra-arterial injection (see below) of aminophylline or caffeine while blood pressure was lower than it was at the time of the "normal" reading; a similar coincidence was encountered in one experiment during a metrazol convulsion and in another during inhalation of oxygen. In the remaining cases the maximum flow was associated with a rise in blood pressure and was produced by intravenous injection of adrenalin in 4, by inhalation of oxygen in 2, and by inhalation of nitrogen in one, while in 3 it was encountered during the "normal" period. The highest flow (103 cc. total, 1.13 cc. per gram) was associated with a drop of blood pressure from a "normal" of 170 to 162 mm. during inhalation of oxygen; the "normal" flow was 75 cc. (0.77 cc. per gram). The "minimum" flow readings in 13 cases corresponded with a fall in blood pressure. Of the 6 in which at the time of the minimum flow pressure either was unchanged or elevated as compared with the "normal," 2 were obtained after intra-arterial injection of adrenalin and 2 after similar injection of benzedrine, one was found after intravenous in-

TABLE 1

	CEREBRAL FLOW		B-P	
	cc./min.	*cc./g./min.*		
Average normal	55	0.60	125	19 expts.—carotid flow, basilar tied
Average maximum	69	0.76	131	
Average minimum	25	0.27	109	
Average normal	60	0.63	97	3 expts.—carotid plus basilar flow
Average maximum	77	0.81	131	
Average minimum	42	0.44	86	
Average normal	42	0.45	99	Same 3 expts.—carotid flow only

jection of adrenalin, and one coincided with inhalation of nitrogen. The minimum readings ranged from 13 cc. (0.14 cc. per gram) at a blood pressure of 90 mm. following adrenalin intra-arterially, to 46 cc. (0.5 cc. per gram) with a blood pressure of 88 mm. after nitroglycerine intravenously. In the former experiment the "normal" and "maximum" values were 35 cc. (0.41 cc. per gram) at 104 mm. and 56 cc. (0.66 cc. per gram) at 100 mm.; in the latter the corresponding figures were 73 cc. (0.83 cc. per gram) at 150 mm. and 100 cc. (1.14 cc. per gram) at 152 mm.

The 3 experiments in which flow was measured simultaneously in the carotids and basilar are treated separately because they show that the figures obtained by measuring only the internal carotid streams represent about 70 per cent of the total carried by both carotids and basilar when both are open. The individual figures were 81 per cent, 67 per cent and 63 per cent. The number of observations is small but the existence of this discrepancy as well as its approximate magnitude seem to us to be clearly indicated. If the 70 per cent factor is used to correct the findings in the 19 experiments in which only carotid flow was

measured the average "normal" cerebral blood flow in the monkey becomes 0.86 cc. per gram per minute. This figure we believe to be closer to the actual value than any that has previously been obtained.

The individual "normal," "maximum," and "minimum" figures show that while cerebral blood flow tends to vary directly with the blood pressure, it can also undergo independent variations of considerable size, particularly under the influence of drugs. Since this confirms in another animal and by another method the conclusions derived from previous experiments made in this laboratory (4, 5, 7, 8, 9) we attempted to parallel the earlier studies as far as possible.

2. *Effect of stimulation of the cervical sympathetic nerve.* Satisfactory tests were made 7 times on 4 animals and the results are summarized in table 2. A number of other trials in which mydriasis failed to occur (indicating ineffectiveness of stimulation) or measurements were unsatisfactory, are not included.

TABLE 2

Effects of cervical sympathetic stimulation

EXPT.	STIM.*		B-P		FLOW	
	On	For	From	To	From	To
					cc./min.	*cc./min.*
2-22	R	2′	108	100	39	38
	L	2½′	110	114	42	44
2-24	R	2′	142	142	46	57
	R	2′	140	142	44	46
	L	2′	146	138	50	34
2-26	L	2′	90	80	38	32
6-19	R	3½′	52	52	41	39

* Mydriasis occurred on the stimulated side in every case. In the experiment of 2-22 salivation also was observed.

The results give no indication of a direct effect of any importance, in which they confirm the outcome of our first attempt (6) at measuring total cerebral blood flow. They are very different from those obtained by a thermocouple in the parietal cortex of the cat (4, 9) where cervical sympathetic stimulation regularly gave rise to an indicated decrease in blood flow, but similar to those obtained by the same thermocouple in the medulla of the cat (5), where no such changes were seen. The possibility that reapportionment of the total stream may take place within the brain when the sympathetic nerve is stimulated receives some support from observations made in the last experiment shown in table 2, which is the only one in which we have as yet been able to study this question by separate measurement of the carotid and basilar blood flow. Of the total flow before stimulation (41 cc. per minute), 28 cc. was carried by the carotids, 13 cc. by the basilar. The carotid flow measurements during the stimulation were 27, 26, and 25 cc. The corresponding figures for basilar flow are

13.4, 13.7 and 14 cc. The blood pressure was constant throughout. The change, though slight, is in the direction demanded by the above hypothesis. That the cerebral blood vessels of these animals were capable of constricting is shown by the results obtained with adrenalin and other sympathomimetic agents (fig. 2).

3. *Effect of changes in the blood gases.* Anoxemia was induced by inhalation of 90 or 100 per cent nitrogen. Oxygen (100 per cent) and carbon dioxide (10

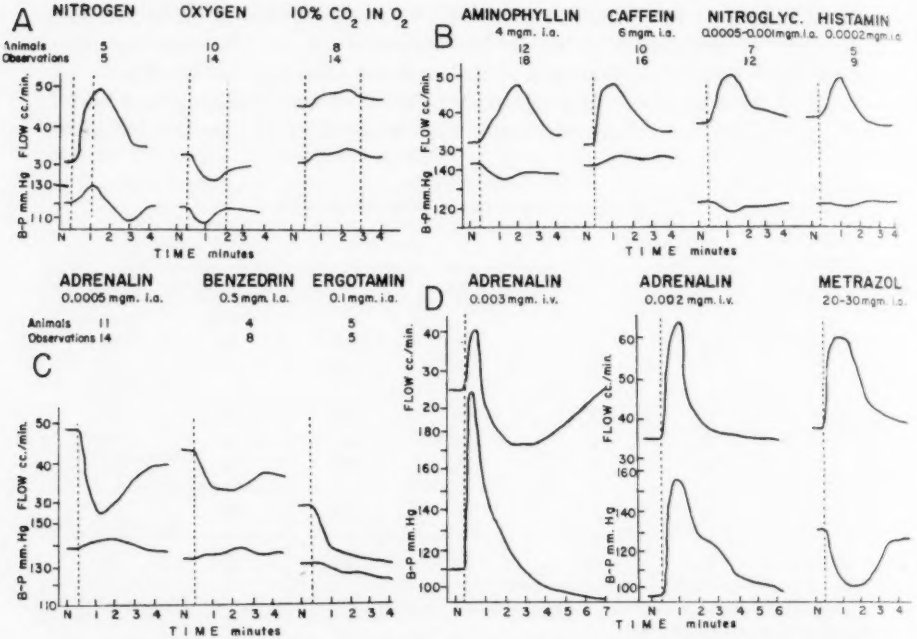


Fig. 2. Average effects on cerebral blood flow (upper) and blood pressure (lower); the numbers of animals and of observations on which curves are based are indicated. *N* signifies the "normal" value and the vertical broken line the time of injection; in A the two broken lines indicate inhalation of pure nitrogen, oxygen, or 10 per cent CO_2 in oxygen. In D the adrenalin curves represent single experiments but the metrazol record is based on the averages of the results of 8 injections in 6 animals. *i.a.* = intra-arterial injection (into the inflow tube of the meter). *i.v.* = intravenous injection.

per cent in O_2) were also tried. The average findings of these procedures are shown in figure 2 A. The individual results did not differ in any important respect from these.

The most important finding here, in our opinion, is the apparent superiority of anoxemia over hypercapnia as a dilator of cerebral vessels in the monkey. The increases in flow brought about by CO_2 were smaller than we had been led to expect by our experience with other animals, while those elicited by anoxemia were much greater. But attention must be called to the following experimental

circumstance, which may modify the deductions to be drawn from this comparison. None of these animals showed more than a slight hyperpnea during anoxemia, due, we believe, to damage to the carotid innervation during the dissection or insertion of the cannulae; that the chemoreceptor reflexes were normally active before these procedures were carried out was shown in a number of cases by vigorous responses to anoxemia or cyanide at the start of the experiment. As has been suggested elsewhere (3), the reflex hyperpnea of anoxemia may lead to sufficient reduction in arterial carbon dioxide tension to cause constriction of cerebral blood vessels. In that event the dilator effect of anoxemia would be antagonized and perhaps overcome, but in these animals no such antagonism was present. The increases in cerebral flow resulting from anoxemia may therefore have been considerably greater than under more nearly normal circumstances.

The effects of the other gases were qualitatively similar to those detected in other animals by other methods. There was a distinct hint of a constrictor action by oxygen, though this was never at all marked. The dilator action of CO_2 was consistent but not very great. In a few cases respiration ceased and asphyxia developed before oxygen could be given by tracheal catheter. In these, cerebral blood flow increased as long as blood pressure did not fall, and there was no sign of cerebral vasoconstriction associated with increased activity of the vasomotor center.

4. *Effects of drugs.* Since this preparation involved exteriorization of the arterial blood supply of the brain and therefore made intra-arterial injections very easy, we frequently availed ourselves of the advantages (see 4) of this method of studying the effects of drugs on the cerebral circulation. Data sufficient at least for tentative conclusions have been secured by intra-arterial injections of adrenalin, benzedrine, ergotamine, histamine, nitroglycerine, caffeine, theophylline, and metrazol. The results are illustrated in figure 2 B and C. They can be summarized by the statement that by intra-arterial injection of adrenalin or benzedrine the cerebral vessels can be constricted quite vigorously, and by similar injection of histamine, nitroglycerine, caffeine, and theophylline they can be dilated. Such changes can occur without any corresponding alteration in blood pressure. Ergotamine decreased cerebral blood flow, but since it also decreased blood pressure in that dosage, and smaller amounts were ineffective, the interpretation is as uncertain as was that of the results of comparable experiments on cats (4). Metrazol caused a pure and usually marked increase in cerebral flow (associated with convulsions, which came on instantly after these intra-arterial injections), while blood pressure fell sharply (fig. 2 D). Acetyl β-methylcholine (Mecholyl) was given intra-arterially 7 times in 3 animals, the dose being 0.0001 mgm. Cerebral blood flow was invariably increased while blood pressure fell slightly; recovery was complete within 3 minutes. The average figures were from 44 to 49 cc. per minute in flow, from 136 to 132 mm. Hg in pressure.

Observations on the effects of these drugs when given by channels other than the intracarotid have so far been infrequent. This is because most of the

drugs, when given in effective dosage, ordinarily have effects so prolonged that only one or two valid tests could be carried out in a single experiment. The experiments were so expensive and difficult that we tried to make as many different observations as possible in each, and intraarterial injections of minimum effective doses were preferred. However, enough intravenous injections were made of adrenalin (10 in 8 animals, excluding injections intended to restore a failing circulation) to show that the constriction of cerebral vessels which intracarotid injection of this drug produces is much less in evidence following intravenous administration (fig. 2 D). In all of these 10 instances there was an increase in flow as blood pressure rose. In 5 the flow descended faster than the pressure and reached a level lower than the starting point though pressure was at or above its control level. In the others the flow came back to a level either the same as or higher than the control. A representative example of each type of response is shown in figure 2 D.

Of the other drugs, only caffeine and theophylline have been given often enough intravenously to justify even tentative conclusions (5 times in 5 animals and 4 times in 3 animals, respectively). Caffeine, in dosage of 10 to 20 mgm., increased cerebral flow slightly in 3 cases, did not change it appreciably in the others; the most marked increase was from 24 to 29 cc. per minute; since blood pressure fell at the same time from 86 to 80 mm. this result appears to be significant. Theophylline (as the ethylene diamine derivative), when injected intravenously in dosage of 10 to 40 mgm., lowered blood pressure quite markedly; cerebral flow was decreased at the same time in 2 cases, increased in the other 2; the most marked increase in flow was from 25 to 30 cc. per minute, associated with a fall in pressure from 106 to 96 mm.—again a significant change.

We have also tried nitroglycerine and insulin by intravenous injection, each in 2 different animals. The former, in 0.5 mgm. dosage, only decreased cerebral flow as blood pressure fell and there was no sign of an effective vasodilator action in the brain. Insulin, in dosage of 5 and 10 units, was used because of the possibility that some of its convulsant effects might be due to violent constriction of cerebral vessels. No trace of any such action was evident (table 3). Blood pressure was lowered as hypoglycemia developed and cerebral flow followed this apparently quite passively. Recovery of the circulation began while the blood sugar was still falling. Convulsions did not appear, doubtless because of the anesthesia.

Posterior pituitary extract (Parke, Davis and Co. obstetrical pituitrin, 10 pressor units per cc.) was injected intra-arterially once in each of 3 experiments, the dose being 0.1 unit (0.1 cc. of a 1 to 10 dilution in saline). The result was an immediate, consistent, and considerable (though transitory) fall in blood pressure accompanied by a parallel decrease in cerebral flow. Thus in one case pressure fell from 116 to 82 mm. within 2 minutes and recovered to 120 by the end of 7 minutes. The corresponding figures for flow were 30, 12, and 28 cc. per minute. The changes in flow seemed to be the result of the fall in blood pressure, but we have not as yet investigated the latter rather surprising result any farther.

Nembutal (Abbott's veterinary solution, 6.5 per cent containing 20 per cent alcohol) was injected intra-arterially in 0.1 cc. dosage (6.5 mgm.) in 3 animals in which narcosis had become too light. The consistent result was a slight but distinct increase in cerebral flow (e.g., from 40 to 46 cc. per minute) associated with a slight fall in blood pressure (from 116 to 110 mm.), with recovery of both within 5 minutes. Other narcotics have not been tried, nor have we as yet attempted to dissociate the effect of the alcohol of this solution from that of the barbiturate.

5. *The basilar-carotid anastomosis.* In the 3 experiments (see table 1) in which we were able to measure flow through the basilar and the internal carotid systems separately, we had an opportunity to determine not only the portion of the total flow carried by each of these, but also the extent to which read-

TABLE 3

	ANIMAL 1			ANIMAL 2			COMMENT
	Cereb. flow	B-P	Blood sugar	Cereb. flow	B-P	Blood sugar	
	cc./min.	*mm. Hg*	*mgm. %*	*cc./min.*	*mm. Hg*	*mgm. %*	
Control	51	118	109	42	108	89	
15 min. after insulin*	33	76	71	23	54	55	Hyperpnea in both animals at this time
30 min. after insulin*	28	60	61	39	100		Hyperpnea ended in both at this time
45 min. after insulin*	36	90		35	94	46	
60 min. after insulin*	39	102	43	31	115	42	Glucose in animal 2 at 65 min.**
75 min. after insulin*	43	108		29	114		Glucose in animal 1 at 80 min.**
100 min. after insulin*	35	100	54	27	112	70	

* Insulin was injected intravenously in dosage of 5 units in animal 1, 10 units in animal 2. Weight of animal 1, 5.5 kilos; of animal 2, 6.2 kilos.

** Glucose was injected intraperitoneally, 25 cc. of 5 per cent solution being given to each animal.

justments can occur under different circumstances. One of these findings, indicating that the carotid component amounts only to about 70 per cent of the actual total when the basilar is closed, has already been mentioned (p. 424). Other observations bearing on this subject are shown in table 4.

These data indicate several points of some importance. One is the variability of the basilar:carotid ratio. In the last experiment cited the basilar was larger than in the other two and it is probable that the ratio here (80 per cent) is quite exceptional. Furthermore, the basilar arteries in all three of these animals were larger than they were in three others in which the vessel was too small for cannulization. For these reasons we do not think it advisable to venture, from the data now available, any deduction other than that the basilar contributes a highly variable portion of the total cerebral flow. More definite conclusions can however be derived on another point, viz., the extent to which the flow

through each system is increased when the other is closed. The increase in basilar flow resulting from carotid occlusion was much greater, in two of the three experiments, than the increase in carotid flow resulting from basilar occlusion. This is to be expected in view of the relative sizes of the two sets of vessels and the volumes carried by them. It is noteworthy in this connection, however, that even the greatest percentile increase in basilar flow (90 per cent) meant that when the carotids were closed the total cerebral flow was 21 cc. per minute, which was only 40 per cent of the amount previously carried by both systems (53 cc.). In the same animal the carotids carried 43 cc. when the basilar was closed, and this, although it was an increase only of 10 per cent in carotid flow, nevertheless amounted to 81 per cent of the previous total.

TABLE 4

EXPT.	FLOWS—BOTH OPEN				CAROTID FLOW ON BASILAR OCCLUSION				BASILAR FLOW ON CAROTID OCCLUSION				CONDITIONS
	Car.	Bas.	B-P	Bas./Car.	From	To	Incr.	B-P	From	To	Incr.	B-P	
	cc./min.	*cc./min.*		*per cent*	*cc./min.*	*cc./min.*	*per cent*		*cc./min.*	*cc./min.*	*per cent*		
6-19	42	11	96	26	39	43	10	84	11	21	90	98	Control—O_2 by tracheal catheter
	37	11	80	30	39	43	10	82	11	18	64	82	Same
	28	13	48	46	28	33	18	52	13	17	30	52	After caffeine, theophylline, and nitroglycerine i.v.
6-23	54	23	140	43	26	33	27	100	23	35	52	152	Control (basilar occlusion later than others)
6-24	24	19	70	80	24	27	13	71	19	22	16	72	Control—O_2 by tracheal catheter
	22	18	72	82	24	29	21	73	18	22	22	74	After adrenalin and caffeine i.v.
	27	17	69	63	23	27	17	67					Later—follows caffeine i.a.

At the other extreme, the smallest percentile increase in basilar flow on carotid occlusion (16 per cent) brought the total flow to 22 as compared with 43 cc. or 51 per cent, and the corresponding increase in carotid flow on basilar occlusion (to 27 cc.) amounted to 65 per cent of the original total. These figures serve to show the order of magnitude of the readjustments brought about through the circle of Willis in these particular animals. The individual variations were so great as to suggest that the consequences of occlusion of these vessels in any given subject can scarcely be predicted.

SUMMARY AND CONCLUSIONS

The volume of blood flowing into the brain through the internal carotids has been measured in 19 monkeys anesthetized with nembutal. The average figure is 0.60 cc. per gram per minute at an average blood pressure of 125 mm. Hg.

Direct measurements of flow through the internal carotids and the basilar indicate that this figure probably represents only about 70 per cent of the total normal flow. The corrected value therefore is 0.86 cc. per gram per minute.

Stimulation of the cervical sympathetic nerve produced no significant alterations in cerebral flow, though in one experiment results suggesting a redistribution of blood were obtained.

Anoxemia and hypercapnia both increased cerebral flow and increased oxygen tended to decrease it. The effects of anoxemia were more striking than those of CO_2 and much more marked than was expected from comparable experiments on other animals.

Given by intracarotid injection and in minimum effective dosage, adrenalin and benzedrine produced consistent and rather striking decreases in cerebral flow and caffeine, theophylline, histamine, and mecholyl produced comparable increases. Ergotamine and pituitary extract caused a decrease in both flow and blood pressure when similarly given.

When given intravenously adrenalin increased cerebral flow as blood pressure rose; when pressure fell flow fell at the same rate in half the cases, at a faster rate (leading to a definitely subnormal flow) in the others. Caffeine and theophylline, similarly administered, sometimes increased flow distinctly, although blood pressure fell, but nitroglycerine had no such effect.

In 3 experiments in which flow through the basilar and internal carotid systems was measured simultaneously, the part contributed by each to the total flow varied widely (basilar: carotid ratio 26, 43 and 80 per cent). Flow through each system increased when the other was closed but the magnitude of the readjustments thus brought about was subject to such great individual variations as to suggest that the quantitative consequences of occlusion of any of these vessels cannot be predicted.

REFERENCES

(1) Ask-Upmark, E. Acta Psychiat. et Neurol. Supp. VI, Copenhagen, 1935.
(2) Gregg, D. E., W. H. Pritchard, R. W. Eckstein, R. E. Shipley, A. Rotta, J. Dingle, T. W. Steege and J. T. Wearn. This Journal **136:** 250, 263, 1942.
(3) Schmidt, C. F. and J. H. Comroe, Jr. Ann. Rev. Physiol. **3:** 168, 1941.
(4) Schmidt, C. F. and J. P. Hendrix. Trans. Assoc. Res. Nerv. and Ment. Dis. **18:** 229, 1938.
(5) Schmidt, C. F. and J. C. Pierson. This Journal **108:** 241, 1934.
(6) Schmidt, C. F. Ibid. **84:** 202, 1928.
(7) Schmidt, C. F. Ibid. **102:** 94, 1932.
(8) Schmidt, C. F. Ibid. **110:** 137, 1934.
(9) Schmidt, C. F. Ibid. **114:** 572, 1935.
(10) Soskin, S., W. S. Priest and W. J. Schultz. Ibid. **108:** 107, 1934.
(11) Wagoner, G. W. and A. E. Livingston. J. Pharmacol. and Exper. Therap. **32:** 171, 1928.
(12) Wolff, H. G. Physiol. Rev. **16:** 545, 1936.

THE CYTOLYTIC EFFECT OF SAPONIN ON THE WALLS OF VESSELS[1]

ERIC PONDER AND CHESTER HYMAN

From The Nassau Hospital, Mineola, N. Y., and The Department of Biology, Washington Square College, New York University

Received for publication September 12, 1942

In a study of the activity of hemolysins *in vivo*, Ponder, Hyman and White (1941) found that lysins such as saponin, when perfused through an organ, disappear from the perfusion fluid, presumably as a result of combining, as cytolysins, with the structures lining the vessel walls and with other tissue cells. The purpose of this paper is to relate the quantity of lysin taken up with the cytolytic effects produced, these being measured by the rate of edema, the diffusion of hemoglobin through the injured vessel walls into the intercellular spaces, and other indications of alteration in the permeability of the vessels.

1. *Edema formation.* The effect of lysins on the rate of development of edema was determined by the method of Hyman and Chambers (1942). Essentially, the method involves the perfusion of frog legs (Rana pipiens) through a cannula inserted into the lower end of the aorta, the perfusion fluid passing into the vascular system of the muscles and skin, and out through the renal portal system and kidneys, to escape through the cut end of the vena cava. A mixture of 1.5 per cent CO_2 and 98.5 per cent O_2 under pressure was used to drive the fluid from the storage reservoirs and to aerate the solutions properly. The perfusion pressure was maintained at 20 mm. Hg by means of a mercury blow-off. The cannulated preparation is placed in a light cylindrical moist chamber suspended by a helical phosphor-bronze spring. As the legs become more and more edematous during a perfusion, their increased weight causes an extension of the spring, and this is continuously recorded by means of a lever writing on a slowly moving drum. The spring should be sufficiently sensitive to record the passage of single drops as they fall from the preparation, thus acting as a drop recorder; in this way changes in the vascular caliber as well as in edema rate can be observed.

The perfusion fluid used was a 0.5 per cent solution of Eastman Purified Calfskin Gelatin in frog Ringer buffered at 7.65.[2] The lysins were prepared in various concentrations in this fluid. In determining the effect of any concentration of saponin we proceeded as follows: The preparation was perfused with the perfusion fluid for about 1 hour in order to find the rate of edema formation with this fluid alone. During this period the weight curve presents a constant slope. The solution containing saponin was then started through the cannula, the commencement of the perfusion with lysin being signalled by mixing a small

[1] This work was carried out with the aid of a grant to one of us (E.P.) from the American Association for the Advancement of Science.

[2] This concentration of gelatin has a lower colloid osmotic pressure than that of frog plasma, and was used in order to assure a measurable edema rate during the initial period of perfusion with the fluid alone.

amount of a non-toxic dye with the first small volume of the fluid containing lysin, and observing the time of appearance of the first colored drop. For the first few minutes of the perfusion with lysin, the slope of the weight curve remains the same as before, but at the end of this latent period it begins to increase, at first slowly and then more rapidly. This is an indication of an increasing permeability of the vessel walls. After attaining a maximum slope, the weight curve begins to flatten out; this is partly the result of a vasoconstriction produced by the saponin, as shown by the relative infrequency of the outflowing drops. In this investigation we are concerned with the weight curve only up to the point of its maximum slope.

The rate of increase in weight of the preparation, i.e., the rate of edema formation, is a measure of the permeability of the vessel walls, since it represents the transfer of a quantity of fluid dQ in time dt. The permeability in the absence of lysin is accordingly measured by the constant slope of the weight curve during the first hour of perfusion. Similarly, the permeability at any time after the introduction of the lysin is measured by the slope of the curve at that time, and the effect of the lysin on the permeability is conveniently measured by the ratio r of that slope to the constant slope obtained during the preliminary perfusion. Making the same kind of assumptions as made for the action of lysins on red cells (Ponder, 1941), let us think of the permeability in the absence of the lysin as involving the passage of fluid through pores occupying a total surface A_1 of the vessel wall, and of the lysin as increasing the number or size of the pores so that the additional surface A_2 is involved. A_2 will then be proportional to $(r - 1)$, which again will be proportional to the amount of lysin used up in breaking down or otherwise transforming the surface of the vessel walls to form the new pores. Further $(r - 1)$ should be some function of c_o, the concentration of lysin in the perfusion fluid, and the kind of function which it turns out to be experimentally is shown in table 1 and in figure 1, in which the maximum value of $(r - 1)$ found for each concentration is plotted against c_o. Each value is the average of from 3 to 8 individual determinations. Similar increases in edema rate have been obtained with sodium taurocholate (1 in 2,500), but, as is well known, this lysin is not so suitable for quantitative work as is saponin.

Figure 1 shows that the relation of $(r - 1)$ to c_o is substantially linear, the intercept on the ordinate, $c_o = 8.5\ \gamma$/ml., corresponding to a quantity of lysin just too small to produce a change in permeability. This quantity measures the lower limit of the frequency distribution of resistances in the vessel walls to breakdown by the lysin (cf. Ponder, 1941, fig. 3). The linearity of the relation shows that both A_2, the area transformed so as to become permeable, and the amount of lysin used up in the transformation, are proportional to c_o, a result arrived at independently below, and familiar because it is met with in other related systems, such as those in which saponin produces cytolysis of white cells (Ponder and Macleod, 1936) or enters into combination with red cell ghosts (Ponder, 1935).

We expected to find the latent period, i.e., the time between the arrival of the lysin at the preparation and the time at which the first increase in edema rate

was observed, to be smaller for high concentrations than for low ones. This may indeed be so, but the experiments do not show it, probably because the concentration range is relatively small and the experiments show so much individual variation.[3] We also expected to find the maximum increase in weight after

TABLE 1

DILUTION 1 IN	c_0	$(r-1)$	SAPONIN SPACE	U
	$\gamma/ml.$			γ
20,000	50	5.0	5.3	1900
35,000	29	2.5	3.5	1220
40,000	25	1.8	2.9	980
50,000	20	1.5	2.5	760
60,000	17	1.1	1.9	620
100,000	10	0.2	1.0	350

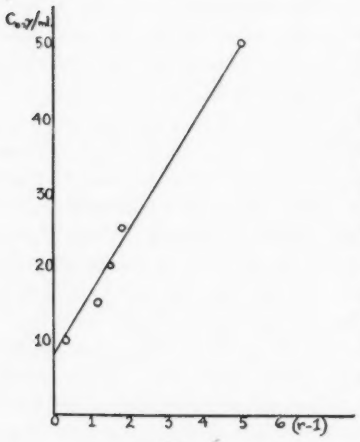


Fig. 1

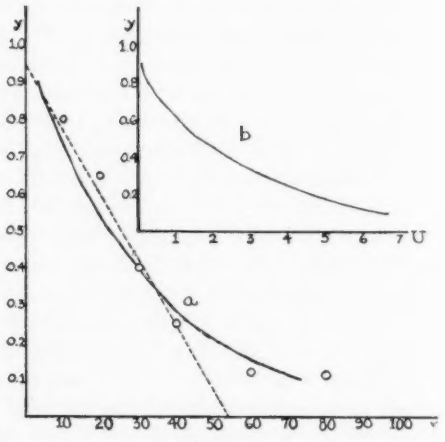


Fig. 2

Fig. 1. Increase of edema rate (abscissa) plotted against concentration of saponin in perfusing fluid (ordinate) for perfusions of 30 minutes' duration.

Fig. 2. Curve a, experimental curve for fraction of lysin taken up, y, plotted against v, the volume of perfusion fluid passed through. Dotted line, Ponder, Hyman and White's approximation. Curve b the integral of curve a (equation 4).

[3] The variability of the material in these experiments is considerable, and we have been unable to eliminate it. An average value of r, such as the 2.5 given for 20 γ/ml., for example, is subject to a variation of ±1.0, and occasionally experiments have had to be rejected altogether because of the extreme vasoconstriction produced by the lysin. The variability in the diffusion of hemoglobin experiments is smaller, amounting to ±10 per cent, but about one out of every four experiments had to be rejected for technical reasons, the principal ones being unequal perfusion rates in the two legs and the appearance of extreme vasoconstriction. The experiments on the uptake of lysin are the best of all, and the great variations in the perfusion rate found by Ponder, Hyman and White in their kidney perfusions were almost completely absent. R. pipiens is better material for all these types of experiment than R. catesbiana.

perfusion with the lysin to be greater in high lysin concentrations than in low ones. The experiments show almost the reverse, the average increase in weight from the beginning of the perfusion with saponin to the maximum of the weight curve being 16, 17.8 and 26.9 per cent of the initial weight for the three concentrations 29, 20 and 17 γ/ml. This is undoubtedly due to the powerful vasoconstriction produced by the higher concentrations of lysin, this preventing a true maximum being reached.

2. *Diffusion of hemoglobin.* Danielli (1941) has described a method for measuring the volume of the vascular system of a muscle by perfusing it with a hemoglobin solution. If a lysin such as saponin is added to the perfusing fluid, cytolysis occurring in the vessel walls allows the hemoglobin to diffuse out of the vascular system into intercellular spaces and perhaps into the muscle cells themselves. The extent of this steadily increasing "saponin space" can be taken as a somewhat arbitrary measure of the amount of destruction produced by the lysin at any time during the perfusion.

The experiments were carried out along lines suggested by Danielli's paper. The hind legs of frogs (R. pipiens) weighing about 50 gram were perfused through the aorta just above the bifurcation, the perfusion being begun within 3 minutes of the pithing of the animal. The vessels were first washed out for 15 minutes with a 2 per cent gelatin in frog Ringer, and were then perfused with a 3 per cent solution of hemoglobin prepared by hemolysing sheep red cells with water, adding salts to bring the salt concentration to that of frog Ringer, centrifuging, and filtering. This perfusion was usually continued for 30 minutes. At the end of this time one common iliac artery was tied off, and the gastrocnemius of the leg on that side was used as a control. The remaining leg was perfused for another 30 minutes with a hemoglobin solution of the same concentration, but containing saponin in known amount, e.g., 20 γ/ml. or 1 in 50,000. The higher concentrations of saponin tend to produce vasoconstriction, which can be off-set to some extent by increasing the perfusion pressure, initially 20 mm. Hg. The perfusion fluids were kept continuously oxygenated.

The gastrocnemii were removed, rinsed, dried with blotting paper, and weighed. Each was then minced and extracted in 5 ml. of distilled water for 1 hour. The extracts were cleared of particles by centrifuging and, after adding an equal volume of 2 per cent HCl and allowing to stand for 30 minutes, the amount of hemoglobin present was found with a photoelectric colorimeter. A correction was made for the effect of the light-scattering substances which are extracted by this procedure from unperfused muscle, and another correction, arrived at in a similar way to that described by Danielli, was made for the incomplete recovery of the hemoglobin from the minced muscles. We have found more variation in the corrections required than Danielli reports, but experience with the method of making them has satisfied us that they are good enough for our purpose.

The results of the experiments can be summarised under 3 heads. *a.* The volume of the vascular system of the frog gastrocnemius perfused with 3 per cent hemoglobin in Ringer is 4.7 per cent of the muscle volume, with a variation

of ±0.6 per cent from frog to frog. This confirms Danielli's result. *b.* When saponin is added to the perfusion fluid, its cytolytic effects enable the hemoglobin to diffuse out of the vascular system into a steadily increasing "saponin space." Expressed as a multiple of the original volume of the vascular system, the saponin space found after a fixed time is an approximately linear function of the lysin concentration. This is shown in table 1, which gives results for perfusions of 30 minutes' duration (22 expts.). Similar results, not shown in the table, have been obtained for sodium taurocholate. *c.* As the perfusion with any one concentration continues, the saponin space increases towards a limit, no doubt related to the limit of extensibility of the tissues. At first the increase is approximately linear with time, but later on it becomes slower. The increase in the saponin space with time presumably results from the continued cytolytic action of the saponin, first on the vessel walls, enabling the hemoglobin to penetrate into extravascular spaces, and later on the boundaries of these spaces, enabling still further penetration to take place.[4]

3. *Uptake of lysin by perfused legs.* The extent to which the cytolysin saponin is taken up by the tissues during these perfusions can be found by using various concentrations for perfusion and determining by means of a hemolytic titration the concentrations which appear from time to time in the perfusates (Ponder, Hyman and White, 1941).

Weighed frog hind limbs (R. pipiens) were perfused through the aorta with saponin in dilutions of from 1 in 20,000 to 1 in 100,000 in frog Ringer. The vessels were first washed out for 15 minutes with frog Ringer, after which the saponin perfusion was begun under a pressure of 20 mm. Hg and continued for from 1 to 3 hours. Successive 5 ml. samples of the perfusate were received into vials. The time required for the collection of each was recorded, the effects of vasoconstriction being offset, so far as possible, by increasing the perfusion pressure. The concentration of saponin in each successive 5 ml. sample was subsequently determined by a hemolytic titration.

If we plot y, the fraction of the initial concentration c_o removed as a result of combination with the tissues at any time t, against v, the volume of the perfusate which has passed through, we get a curve such as that shown in figure 2, *a*. The curve, convex to the v-axis, is quite flat; the position of the points at the lower end is somewhat uncertain because of the lower precision of the hemolytic titrations in this region, and the whole shape of the curve is liable to be affected by lack of constancy in the perfusion rate.[5] But if the lysin, supplied at a con-

[4] Before Danielli's paper appeared, we had carried out a number of perfusions with the dye Evans' Blue, before and after the addition of saponin. This dye remains confined to the vascular bed in the absence of the lysin, and perfused muscle stains a pale blue. When saponin is added, the dye escapes through the cytolysed vessel walls, and the muscle becomes intensely blue. We were unable to make these experiments quantitative because we could not extract the dye from the muscle.

[5] In their experiments on the uptake of lysin by kidney tissue, Ponder, Hyman and White obtained relations similar to these, but they used a straight line with a small intercept on the y-axis as an approximation to the relation between y and v. In the light of the experiments in this paper, this is not admissible even as an approximation, because the

stant rate, combines with a component S_o (which can be put, for convenience, as = 100) of the vessel wall and transforms S of it, the rate of disappearance of lysin should be proportional to the amount of surface left untransformed, or

$$-dy/dt = -dy/dv = k(S_o - S) = k(y) \quad \ldots\ldots (1)$$

Integrating,

$$v = 1/k \,.\, \log S_o/y \quad \ldots\ldots (2)$$

which is the equation of the experimental curve obtained by plotting y against v, as in figure 2, *a*, where S is put = 100 for convenience, and in which k = 0.0313. If the perfusion is allowed to continue until the combining power of the tissues is exhausted, the total amount of lysin taken up will be

$$U = 1/k \int \log (S_o - y).dy \quad \ldots\ldots (3)$$

$$= 1/k \{ \log S_o - \log y - S_o/y + 1 \} \quad \ldots\ldots (4)$$

Curve *b* in figure 2 shows the value of this integral for various values of y, S_o being again put = 100. In practice, U is found directly from the experimental curve (e.g., figure 2, *a*) by graphical methods.

From (4), if y and U are both measured in γ/ml., we should have U/c_o = a constant if S_o is constant, as it should tend to be for any one kind of material. Thus U should be proportional to c_o, and this is what is found in a series of perfusions of frog legs with saponin (table 1, last column). This means that while the uptake capacity of the reaction surface is still unexhausted, an approximately constant fraction of c_o is taken up per unit surface. Such a relation is similar to that found by Ponder and Macleod (1936) for the amount of lysin taken up by suspensions of leucocytes as a function of time and of lysin concentration: "The disappearance of lysin is rapid at first, and tends to reach a final value asymptotically (cf. fig. 2, *b*), the amount which finally disappears being an almost linear function of the amount initially present". Similar relations have been obtained for the uptake of saponin and other lysins by red cell ghosts (Ponder, 1935), by kidney tissue (see footnote 4), and, indirectly, in the first section of this paper. The relations, in fact, seem to be quite general.

It remains to relate the quantity of saponin which disappears in the course of a perfusion to the effect which it produces, and here we must be content with an order of magnitude only. For the concentration 20 γ/ml. the maximum edema

point $y = 100$, $v = 0$ has absolute precision and must lie on the curve relating y and v. Looking back over the kidney data, it is clear that in most of the cases in which a sufficient experimental range was covered the relation between the variables is a curve convex to the v-axis. The approximation $U = c_o \cdot ab/2$, made on the basis of the linear relation, nevertheless gives U/b as a nearly constant fraction of c_o (Ponder, Hyman and White's table 2), which is analogous to what we find here. Note: The expression of y as a percentage rather than as a fraction of c_o in Ponder, Hyman and White's paper has resulted in an error in an index on p. 21. The amount of saponin combined with 1 gram of kidney tissue should be 130, and the factor of loss should be 3.4 (10^{-2}). This makes the effectiveness of a lysin *in vivo*, as compared with its effect *in vitro*, 1/2000th.

rate $r = 2.5$ is reached after about 30 minutes, and after the same time we have a saponin space of about 2.5 times the initial vascular volume of the muscle. Now for this concentration and time, $U = 420$. Taking 3μ as the radius of a capillary, which is a minimum value in the presence of a capillary poison, and assuming that the capillary bed makes up 5 per cent of the muscle volume, we find that 420 γ of saponin would form a layer on the capillary walls about 120 Å. thick, corresponding to something less than 10 saponin molecules in depth. For the changes in edema rate and in saponin space measured here, the quantities of lysin involved are accordingly of the same order, per unit area of surface, as the quantities involved in bringing about breakdown of the red cell membrane.

SUMMARY

When frog muscle is perfused with saponin or bile salts in frog Ringer, the permeability of the vessel walls is increased, and this results in an increased rate of edema formation. If the muscle is perfused with a solution of hemoglobin to which saponin or bile salts have been added, the hemoglobin, which in the absence of the lysins remains in the vessels, escapes through the vessel walls and appears in the extravascular spaces. While these effects are being produced, the lysins disappear, in part, from the perfusion fluid, being taken up by the vessel walls and other tissue cells. Quantitative determinations of the rate of uptake of the lysins and of their effects on permeability show that the kinetics of the cytolytic process are similar to the kinetics of hemolytic processes. The quantities of saponin involved in producing these changes in permeability are such as would cover the walls of the vascular system of the muscle with a layer of lysin less than 10 molecules thick.

REFERENCES

DANIELLI, J. F. J. Physiol. **100:** 239, 1941.
HYMAN, C. AND R. CHAMBERS. In press.
PONDER, E. Biochem. J. **29:** 1263, 1935.
J. Gen. Physiol. **25:** 247, 1941.
PONDER, E.; C. HYMAN AND L. WHITE. This Journal **132:** 18, 1941.
PONDER, E. AND J. MACLEOD. J. Gen. Physiol. **20:** 267, 1936.

EFFECT OF HYPOPHYSECTOMY AND OF PURIFIED PITUITARY HORMONES ON THE LIVER ARGINASE ACTIVITY OF RATS[1]

HEINZ FRAENKEL-CONRAT, MIRIAM E. SIMPSON AND HERBERT M. EVANS

From the Institute of Experimental Biology, University of California, Berkeley

Received for publication September 14, 1942

The rôle of the pituitary in controlling nitrogen metabolism has long been recognized. Thus the growth stimulating action of pituitary extracts was shown to be associated with nitrogen retention, while the rate of protein breakdown appears to depend upon the adrenocorticotropic action of the hypophysis. Both the protein anabolic action of growth hormone and the catabolic action of adrenocorticotropic hormone (ACTH) influence the rate of urea excretion and it therefore appeared indicated to investigate the effect of various hormones on the enzyme systems involved in the formation of urea. Arginase was chosen as the first enzyme to be studied, since it seemed to be more specifically connected with the formation of urea than amino-acid oxidases, transaminases, etc. Even if the Krebs cycle for the formation of urea should not withstand criticism without modification, the involvement of arginine and arginase in the formation of urea appears well established.

Studies of the hormonal control of liver arginase were initiated by Lightbody and Kleinman (1) who investigated the action of thyroxin on this enzyme system. In view of the fact that thyroxin may show variable effects in regard to nitrogen metabolism, dependent on the dose and other endocrine factors, its lack of a clear-cut action on the arginase activity is not too surprising. It was hoped that certain pituitary factors might reflect their unequivocal action on nitrogen metabolism in more consistent changes in liver arginase concentration. This appeared to be the case when the adrenocorticotropic hormone (ACTH) was found to increase liver arginase activity markedly (2). Confirmation of this finding, which has recently been extended to certain adrenal steroids (3), will here be presented. Besides, an action opposite to that of ACTH will be demonstrated to be exerted by other pituitary hormones, primarily the growth hormone. Furthermore, the effect of hypophysectomy which was found to lead to a decrease in the arginase activity of rat's livers, will be discussed.

EXPERIMENTAL CONDITIONS, METHODS AND MATERIALS. Rats of the Long-Evans strain of both sexes and various age groups were used for these studies. For most experiments immature females were employed, many of which were hypophysectomized at the age of 26 to 28 days. For another series of experiments, 2 to 3 month old males were used, either from the day following the operation, or 2 to 4 weeks postoperative. They were treated with hormone solutions

[1] Aided by grants from the Board of Research of the University of California and the Rockefeller Foundation, New York City, and Parke, Davis and Company, Detroit, Michigan. We wish to acknowledge assistance from the Works Projects Administration, Project no. OP-65-1-08, Unit A-5.

for various time periods, ranging from 4 hours to 15 days, receiving one or two intraperitoneal injections daily. In many experiments three injections were given during the 24 hours preceding autopsy. All rats were fasted, either for 24 hours or 7 to 8 hours, before autopsy. Since the arginase content of untreated rats was found to differ for the two fasting periods, the data were grouped according to the length of the fast. Preceding the fast the animals were fed ad libitum, with the exception of a few experiments in which both hypophysectomized rats and their unoperated controls were fed equal amounts of diet by stomach tube.[2]

For the determination of arginase, the livers were removed under sodium amytal anesthesia and immediately weighed and placed on ice. In the case of hypophysectomized rats, the sella was then searched for residual pituitary tissue and only the livers of completely hypophysectomized rats were used. In a number of experiments, individual livers were analysed, but in general about one-third of each liver was cut off, these fractions then being pooled according to groups and analyses of the pooled livers performed in duplicate. For that purpose the liver tissue is broken up and finely suspended in a hundred fold of distilled water by means of a Waring Blendor, 1 or 2 minutes being generally used, at the low speed. The suspensions are then centrifuged and 0.5 cc. of this liver extract added to 1 cc. 2.5 percent arginine carbonate solution and 1.25 cc. 0.1 M pH 9.5 glycine sodium hydroxide buffer. Then 1 cc. water is added and the tubes (pyrex test tubes, graduated at 5 cc.) placed in a water-bath at 38 to 39°C. for 60 minutes. They are then immediately transferred into a boiling waterbath for 15 minutes, to inactivate the enzyme. The amount of urea formed by the enzyme action is determined by the use of xanthydrol. For this purpose the digests are made up to the 5 cc. mark and 2 cc. is pipetted into 15 cc. centrifuge tubes. To this is added 7 cc. glacial acetic acid and 0.2 cc. 10 percent methylalcoholic xanthydrol solution 5 times at 10 minute intervals. The solutions are occasionally stirred with glass rods during this time and again after a few hours standing, to ensure complete crystallization of the urea-xanthydrol condensation product. The following day these precipitates are centrifuged off, washed with 3 cc. methanol, again centrifuged and drained. They are then redissolved by the addition of concentrated sulfuric acid, quantitatively washed into micro-Kjeldahl flasks and their nitrogen determined. The unitage of the enzyme concentration per gram liver is calculated from these urea-nitrogen values according to Edlbacher (5). Since activators are neither added nor removed from the liver extracts, the determination gives a measure of arginase activity rather than of the concentration of the enzyme in these livers. Whenever in this discussion arginase content or concentration is mentioned, reference is made to the naturally activated arginase or the arginase activity per gram liver tissue.

A great number of duplicate digests with the same liver extract, as well as duplicate urea determinations after digestion gave values which generally were within 2 to 3 percent, differing rarely by as much as 10 percent. If the liver

[2] A diet modified by W. Marx (4) to assure easy passage through a narrow catheter tubing.

extracts were kept at room temperature for a few hours before use, or were stored at 2 to 5°C. for 2 days, their arginase activity was found 10 to 20 percent lower than in the fresh tissue. Therefore all analyses were generally performed immediately following autopsy.

Through analyses of individual livers of similarly treated rats a measure of the variations that are to be expected within groups was obtained. Statistical analysis of these results indicated that in groups of 4 rats a positive difference of 33 percent or a negative one of 25 percent was significant ($P<0.05$) whereas differences smaller than 20 percent were not significant in groups of this size. Assuming variations to be similar in experiments where they were not determined owing to analyses being performed on the pooled livers, differences exceeding 20 percent always led to a repetition of the experiment and final conclusions were only drawn when such or greater differences were obtained in a series of experiments.

The hormone preparations were the same as those used in other recent metabolic studies (6), part of the data being obtained from the same rats. The adrenocorticotropic (ACTH), growth, lactogenic and interstitial cell stimulating (ICSH) hormones contained probably no more than one percent of any one of the other hormones, while thyrotropic and follicle stimulating fractions may have been contaminated by 10 to 15 percent of ICSH. The authors are indebted to C. H. Li, W. Marx, W. R. Lyons and J. Fraenkel-Conrat for kindly supplying many of these preparations.

Results. *Effect of hypophysectomy on liver arginase.* A comparison of the arginase activity of the livers of similar untreated normal and hypophysectomized rats indicated a great difference, the enzyme concentration in normal rats being about twice as high as in the operated rats (table 1). In view of the well established dependence of arginase concentration upon diet and food intake (7), it appeared essential that this factor be carefully controlled before the difference between normal and hypophysectomized rats could be attributed to a specific action of the pituitary. To that end part of a group of immature female rats was hypophysectomized; both operated and normal control rats were then fasted for 24 hours; for the ensuing five days they received as much diet as was voluntarily consumed by the hypophysectomized rats and the next four days all were fed equal amounts by stomach tube, followed by a 24 hour fast preceding autopsy. Also under these conditions, the operated rats showed arginase activities which were exactly half of those of the normal rats (average of four hypophysectomized rats was 930 units; of five normal rats 1860 units per gram liver). This was so, notwithstanding the fact that a stasis in body weight was produced by the inanition of the normal rats which led to similar final weights in these as in the operated rats. Since the livers are in general slightly heavier in proportion to the body weight, in normal than in hypophysectomized rats, these differences in arginase activity are even greater when expressed as total arginase per 100 gram rat. It must therefore be concluded that hypophysectomy leads to a striking decrease in liver arginase which is independent of any possible effect of the voluntary inanition on the part of the operated rats.

Effect of sex, age and fasting period on liver arginase. Values for the liver arginase activity of about 60 groups of rats of various types are summarized on table 1, for the purpose of comparison. It appears that immature male rats

TABLE 1

Liver arginase of untreated rats

TYPE OF RAT, AGE AT AUTOPSY	NO. OF RATS OR GROUPS*	LENGTH OF FAST	ARGINASE UNITS PER GRAM LIVER	
A: Normal rats				
		hours		
Immature male, 24 days	3 rats	24	1560	*1570*
	3 rats	24	1570	
Immature female, 30 days	5 rats	24	2280	
36 days	5 rats	24	1860	*2010*
44–46 days	5 rats	24	1900	
30 days	3 rats	8	2960	*2840*
	3 rats	8	2720	
Plateaued female, 5–7 months	5 rats	24	2130	*2200*
	1 rat	24	2540	
B: Hypophysectomized rats				
Female, 26–28 days at op., 1–15 weeks p.o.	18 groups	24	1000	±210†
Male, 2–3 months at op., 2–4 weeks p.o.	7 groups	24	1020	±170
Plat. female, 1 month p.o.	8 rats	24	910	
Female, 26–28 days at op., 9–11 days p.o.	13 groups	8	1320	±240
Female, 26–28 days at op., 29 days p.o.	5 rats	8	950	
Female, 26–28 days at op., 2–3 months p.o.	3 rats	8	1150	
C: Adrenalectomized rats‡				
Female, 26–28 days at op., 18 days p.o.	4 rats	24	580	*580*
	2 rats	24	570	

* From 3–8 rats per group.

† Standard deviations for small samples calculated according to: $\sigma = \sqrt{\frac{\Sigma(x^2)}{N-1}}$.

‡ From ref. (3).

(24 days old) show a somewhat lower arginase content than slightly older females (30–46 days old). There does not seem to be any appreciable change with progressing age in the females, the arginase content being similar in the livers of immature and adult plataued rats. These findings are in agreement with the observations of Lightbody who has shown that male rats exceed females

and show increased arginase concentrations only during the period of sexual maturity and activity while females of all age groups showed similar liver arginase contents (8). When the effect of 8 and 24 hours' fasting is compared, the liver arginase concentration is found 25 to 30 percent lower after the longer fast in all types of rats in which relevant data are available (immature females and various kinds of hypophysectomized rats). In view of the loss in liver weight occurring during the 24 hours' fast (about 15 percent), this difference is actually greater when expressed as total liver arginase. Comparison of the enzyme concentration of hypophysectomized animals of different age, sex and postoperative period indicates no differences after 24 hours' fast. After the 8 hours fasting period, two groups which were one to three months postoperative showed lower arginase activities than others which were used one to two weeks after the operation.

Effect of purified pituitary hormones on liver arginase. It has already been reported that adrenocorticotropic hormone (ACTH) increased liver arginase in hypophysectomized rats. This finding has been amply confirmed in rats of various types, with determinations of the arginase after 8 and 24 hours' fasting (table 2, A; 3, A; 5,12–15). The effect was found to parallel other indications of adrenocorticotropic activity, such as increase in adrenal weights (or maintenance when given after hypophysectomy), thymus atrophy and maintenance of carbohydrate stores during fasting. It was found that approximately 1 mgm. of purified ACTH preparations had to be administered daily to produce a significant effect in hypophysectomized rats. In immature normal males, 2 to 5 mgm. hormone were needed. That the effect was independent of the food intake was shown in one experiment in which all animals were fed equally by stomach tube (table 2,A, 5).

Growth hormone was given to a great number of hypophysectomized rats for various time periods (table 4;5). When more than 0.2 mgm. hormone was administered, this principle was found to decrease the liver arginase concentration in all but one experiment. Striking effects could be observed as early as after one day of treatment but doubtful ones after four or five hours. Also in normal plateaued females this hormone markedly decreased the arginase, but was ineffective in immature females at a dose at which also its growth stimulating action was not evident (table 3,B).

Thyrotropic hormone did not affect liver arginase in short term experiments (table 2,B). On the other hand, decreases in liver arginase activity were observed in two experiments in which this hormone was given to hypophysectomized rats for 10 days, starting on the day following operation (table 5,7,8). A low dose of thyroxin appeared to be similarly effective under these conditions (table 5,8a). Lactogenic hormone showed no effect within three or four days (table 2,C). Treatment for 10 to 14 days after hypophysectomy caused variable effects on the arginase concentration (table 2,C; 5,11). A mixture of the two gonadotropins, the follicle stimulating and the interstitial cell stimulating hormone (FSH and ICSH), caused decreases in the arginase activity of hypophysectomized rats in two similar experiments (table 5,9,10).

While the arginase activity has in general been expressed in units per gram liver, it must be kept in mind that various hormones are known to affect liver weights under similar conditions (9). The ACTH in particular, as well as

TABLE 2

Effect of adrenocorticotropic, lactogenic and thyrotropic hormone on liver arginase of hypophysectomized rats, fasted for 24 hours

EXPT. NO.	LENGTH OF TREATMENT	DAILY DOSE	TYPE AND NUMBER OF RATS	LIVER ARGINASE (PER GRAM LIVER)	
				Units	Change
			A: Adrenocorticotropic hormone		
	days	*mgm.*			%
1	2	3.3	3 immat. females, 9 days p.o.	1530	+53
		0.67	3 immat. females, 9 days p.o.	1040	+4
		0.17	3 immat. females, 9 days p.o.	1110	+11
		0.03	3 immat. females, 9 days p.o.	1160	+16
2	3	1.3	3 immat. females, 7 days p.o.	1550	+55
		0.26	3 immat. females, 7 days p.o.	1080	+8
3	3	3.0*	6 immat. females, 3–15 weeks p.o.	1200	+20
4	4	0.25	3 2–3 month old males, 1 day p.o.	880	−12
5	10	1.0*	4 immat. females, 1 day p.o.	1330	+33
6	14	5.0*	5 2–3 month old males, 1 day p.o.	1630	+63
7	14	3.9*	3 2–3 month old males, 1 day p.o.	2700	+170
8	14	3.0*	3 2–3 month old males, 1 day p.o.	2000	+100
9	14	3.0*	4 2–3 month old males, 1 day p.o.	2350	+135
10	14	2.5*	5 2–3 month old males, 1 day p.o.	1800	+80
			B: Thyrotropic hormone		
1	4 hours	1.0	7 plat. females, 5 weeks p.o.	920	−8
2	4 hours	1.0	5 2–3 month old males, 14 days p.o.	1000	0
3	24 hours	1.0	8 2–3 month old males, 14 days p.o.	920	−8
4	24 hours	0.5	7 immat. females, 1 week p.o.	1280	+28
			C: Lactogenic homone		
1	3	3.0	6 immat. females, 3–15 weeks p.o.	1180	+18
2	4	2.5	5 2–3 month old males, 3–15 weeks p.o.	960	−4
3	4	2.5*	4 2–3 month old males, 3–15 weeks p.o.	800	−20
		2.5*	4 2–3 month old males, 3–15 weeks p.o.	1120	+12
4	14	2.0*	5 2–3 month old males, 1 day p.o.	1370	+37
5	14	1.5*	4 2–3 month old males, 1 day p.o.	1800	+80
6	14	1.5*	4 2–3 month old males, 1 day p.o.	1430	+43
7	14	1.25*	5 2–3 month old males, 1 day p.o.	1360	+36

* Half the daily dose given 3 times during the fasting period.

thyrotropic hormone, were found to increase liver weights when given for 10 to 14 days from the day after the operation, while growth hormone is known to delay the growth of the liver when compared with that of the body. Thus both

the increases in liver arginase produced by prolonged administration of ACTH, and the decreases produced by growth hormone (but not those due to the thyroid) would appear even more pronounced were they calculated and expressed in terms of total arginase activity per 100 gram rat.

DISCUSSION. The hypophysis is shown to play a dual rôle in the control of liver arginase activity of rats, just as it is known to do in regard to protein metabolism. The liver arginase increasing action is due to the adrenocorticotropic hormone (ACTH). The evidence that this action is really mediated by the adrenal cortex has been demonstrated elsewhere (3): adrenalectomy caused decreases in liver arginase which were even more marked than those following hypophysectomy, and corticosterone and related steroids were highly effective in increasing liver arginase in normal, hypophysectomized and adrenalectomized rats. This action of the ACTH on liver arginase is in harmony with its established stimulation of protein breakdown and gluconeogenesis.

TABLE 3

Effect of adrenocorticotropic and growth hormone on liver arginase of normal rats

LENGTH OF TREATMENT	DAILY DOSE	TYPE AND NO. OF RATS	LIVER ARGINASE PER GRAM LIVER	
			Units	Change
A: Adrenocorticotropic hormone				
days	*mgm.*			%
3	10.0	3 21 day old males	1930	+23
	5.0	3 21 day old males	1820	+16
	1.7	3 21 day old males	1500	−4
B: Growth hormone				
8	3.0	5 plateaued females	1470	−31
	1.0	5 21 day old females	2150	−4

In contradistinction to this, growth hormone was found to decrease liver arginase in hypophysectomized and normal rats. This also is in harmony with the known action of the growth hormone in decreasing the formation and excretion of urea. The fact that thyrotropic hormone and thyroxin may cause decreases in liver arginase activity becomes understandable in view of their ability to produce nitrogen retention (10) and growth in normal rodents (11) and limited weight gains in hypophysectomized rats, at physiological dose levels (9, 12). However, the lack of a rapid action of thyrotropic hormone on liver arginase does not favor this as the mechanism through which thyrotropic hormone lowers blood urea within a few hours (13). The inconsistent effects of lactogenic hormone may possibly be due to variable degrees of ACTH contamination in some of the preparations. The arginase decreasing action produced by a mixture of the purified gonadotropins (FSH and ICSH) is surprising; no effect was found to be produced by physiological or high doses of the female sex hormones (3). It must be kept in mind, however, that the gonadotropins in these experiments

were administered at very high doses when expressed in gonadotropic unitage; while these hormones do not cause general nitrogen retention, it appears possible

TABLE 4

Effect of growth hormone on liver arginase of hypophysectomized rats, fasted for 24 hours preceding autopsy

EXPT. NO.	LENGTH OF TREATMENT	DAILY DOSE	TYPE AND NO. OF RATS	LIVER ARGINASE (PER GRAM LIVER)	
				Units	Change
		mgm.			%
1	4 hours	1.0	6 2–3 month old males, 3 weeks p.o.	1030	+3
	1 day	1.0	6 2–3 month old males, 3 weeks p.o.	780	−22
	1 day	0.2	6 2–3 month old males, 3 weeks p.o.	940	−6
2	1 day	0.5	7 immat. females, 1 week p.o.	840	−16
	1 day	0.5	7 immat. females, 1 week p.o.	960	−4
	1 day	0.1	7 immat. females, 1 week p.o.	1050	+5
3	1 day	3.0	3 immat. females, 1 week p.o.	840	−16
	1 day	0.75	3 immat. females, 1 week p.o.	800	−20
	3 days	0.5*	3 immat. females, 1 week p.o.	830	−17
	3 days	0.5*	3 immat. females, 1 week p.o.	730	−27
4	3 days	1.5*	6 immat. females, 3–15 weeks p.o.	670	−33
5	3 days	2.0*	3 immat. females, 2 weeks p.o.	570	−43
	3 days	0.5*	3 immat. females, 2 weeks p.o.	530	−47
	3 days	0.1*	3 immat. females, 2 weeks p.o.	1000	0
6	3 days	1.5	3 immat. females, 3 weeks p.o.	850	−15
	3 days	0.5	3 immat. females, 3 weeks p.o.	880	−13
	3 days	0.5	3 immat. females, 3 weeks p.o.	900	−10
	3 days	0.2	3 immat. females, 3 weeks p.o.	1060	+6
7	3 days	2.0	3 immat. females, 3 weeks p.o.	1200	+20
8	4 days	0.25*	3 2–3 month old males, 2 weeks p.o.	810	−19
9†	4 days	1.0	3 immat. females, 2 months p.o.	720	−28
	4 days	0.5	3 immat. females, 2 months p.o.	840	−16
10	5 days	0.5*	3 immat. females, 1 week p.o.	760	−24
	7 days	0.5*	3 immat. females, 1 week p.o.	870	−13

* Half the daily dose given 3 times during the fasting period.
† Forty-four hours' fast preceding autopsy.

that their liver arginase decreasing action may be correlated with the tenfold increases in ovarian and uterine weights occurring in these rats. In general it must be concluded that the effects produced by thyrotropic, lactogenic and gona-

dotropic hormones need further investigation and confirmation before definite conclusions may be drawn concerning their mechanism.

In view of the opposed effects on liver arginase activity exerted by various pituitary principles, the removal of this gland might not be expected to produce a marked effect on this enzyme system. Actually, striking decreases in the

TABLE 5

Effect of purified pituitary hormones on liver arginase of hypophysectomized rats, 26–28 days at operation, fasted for 8 hours preceding autopsy

EXPT. NO.	HORMONE PREPARATION	LENGTH OF TREATMENT	NO. OF RATS	DAILY DOSE*	DAYS P.O. AT ONSET	LIVER ARGINASE (PER GRAM LIVER)	
						Units	Change
				mgm.			%
1	Growth	5 hours	3	0.5	7	1110	−16
		32 hours	3	0.5	7	900	−32
		32 hours	3	0.5*	7	870	−34
2	Growth	3 days	3	3.3	7	1070	−19
3	Growth	3 days	3	1.25	7	1050	−20
		3 days	3	0.25	7	830	−37
		3 days	3	0.05	7	1360	+3
4	Growth	4 days	3	1.6	7	810	−38
5	Growth	15 days	9	0.1	14	860	−35
6	Growth	10 days	4	0.5	1	570	−57
7	Thyrotropic	10 days	3	2.0	1	800	−39
8	Thyrotropic†	10 days	3	1.0	1	1030	−22
8a	Thyroxin†	10 days	4	0.0075	1	1010	−23
9	Gonadotropic‡	10 days	3	2.0	1	710	−46
10	Gonadotropic‡	10 days	4	1.0	1	900	−32
11	Lactogenic	10 days	4	1.0	1	790	−40
12	Adrenocorticotropic	10 days	3	2.0	1	1550	+18
13	Adrenocorticotropic	9 days	3	1.0	2	1990	+51
14	Adrenocorticotropic	9 days	3	0.2	2	1420	+8
15	Adrenocorticotropic	15 days	5	3.0*	14	1920	+47

* Injections once daily in all experiments but 1 and 14. In experiment 1 the same dose was given once in the first two groups, 5 and 32 hours preceding autopsy; in the third group this dose was distributed in 4 injections over 32 hours. In experiment 14 the hormone was distributed over 2 daily injections.

† The oxygen consumption was found maintained at normal or slightly supernormal levels in expts. 8 and 8a respectively, the controls being 30 per cent below normal.

‡ A mixture of half FSH and half ICSH.

arginase were regularly observed following hypophysectomy; through paired feeding experiments, this response was shown not to be due to the low food consumption of hypophysectomized rats. Light was thrown on this question through a comparison of the relative dose levels of the two main "opponents," ACTH and growth hormone, required for the production of changes in liver arginase activity. Of the ACTH about 1 mgm. has to be administered daily to hypophysectomized rats to cause this or any other functional effect, such as

maintenance of carbohydrate stores during fasting, thymus atrophy, etc.[3] This same dose also prevents adrenal atrophy when given from the day following the operation. It can therefore be concluded that the action of ACTH in increasing liver arginase is produced by a physiological dose, i.e., by an amount of hormone equivalent to that secreted by the intact hypophysis. On the other hand, growth hormone is effective in decreasing liver arginase only at doses of 0.1 to 0.5 mgm. daily, although these preparations at one-tenth of this dose level produce marked weight gains in such hypophysectomized rats. It thus appears probable that the normally functioning pituitary gland does not secrete enough growth hormone to balance the action of ACTH in regard to arginase concentration. It then becomes understandable why the overall effect of removing both hormones, i.e., hypophysectomy, causes a fall in arginase concentration. On the other hand, the finding that adrenalectomy leads to lower liver arginases than occur after hypophysectomy (3) can be regarded as further evidence for the existence of a pituitary principle with an action opposed to that of ACTH. The question poses itself whether the pituitary controls nitrogen metabolism through its action on arginase concentration or whether the changes in arginase activity represent secondary adjustments to an altered metabolism. In regard to the rôle of the adrenal, and thus of ACTH, evidence will be presented elsewhere (3) favoring the view that arginase concentration may actually represent one of the points of attack through which this gland accelerates protein breakdown. On the other hand, the finding that growth hormone stimulates growth at levels which are considerably lower than those necessary to produce appreciable changes in the arginase concentration does not favor a similar interpretation for the action of this hormone. It appears here more likely that the arginase is decreased secondarily in response to a lessened need for this enzyme in animals with a positive nitrogen balance.

SUMMARY

1. Hypophysectomy was shown to lead to a marked decrease in liver arginase activity.

2. This process could be reversed by the administration of adrenocorticotropic hormone. This pituitary hormone also increased the arginase activity of the livers of normal rats.

3. In contradistinction to this, growth hormone was found to decrease the arginase activity in hypophysectomized and normal rats.

4. The possible physiological significance of these newly discovered functions of the anterior hypophysis was discussed.

REFERENCES

(1) LIGHTBODY, D. H., E. WITT AND A. KLEINMAN. Proc. Soc. Exper. Biol. and Med. **46:** 472, 1941.

(2) FRAENKEL-CONRAT, H. AND H. M. EVANS. Science **95:** 305, 1942.

(3) FRAENKEL-CONRAT, H., M. E. SIMPSON AND H. M. EVANS. J. Biol. Chem., in press.

[3] Much lower doses (0.01 mgm. daily) only are needed to produce histologically detectable adrenal stimulation.

(4) Marx, W., M. E. Simpson, W. O. Reinhardt and H. M. Evans. This Journal **135:** 614, 1942.
(5) Edlbacher, S. and H. Rothler. Ztschr. Physiol. Chem. (Hoppe-Seyler) **148:** 264, 1925.
(6) Fraenkel-Conrat, H., V. V. Herring, M. E. Simpson and H. M. Evans. In preparation for press.
(7) Lightbody, D. H. and A. Kleinman. J. Biol. Chem. **129:** 71, 1939; Proc. Soc. Exper. Biol. and Med. **45:** 25, 1940.
(8) Lightbody, D. H. J. Biol. Chem. **124:** 169, 1938.
(9) Fraenkel-Conrat, H., M. E. Simpson and H. M. Evans. This Journal **135:** 398, 1942.
(10) Marx, W., D. B. Magy, M. E. Simpson and H. M. Evans. This Journal, **137:** 544, 1942.
(11) Koger, M., V. Hurst and C. W. Turner. Endocrinol. **31:** 237, 1942.
(12) Laqueur, E., E. Dingemanse and J. Freud. Acta brevia neerland **11:** 46, 1941.
(13) Fraenkel-Conrat, J., H. Fraenkel-Conrat and H. M. Evans. This Journal **137:** 200, 1942.

STUDIES ON HEMOCONCENTRATION AND SHOCK FOLLOWING SEVERE HEMORRHAGE[1]

R. E. WESTON, MARTHA JANOTA, S. O. LEVINSON AND H. NECHELES

From the Samuel Deutsch Serum Center and from the Department of Gastro-Intestinal Research of Michael Reese Hospital and from The Department of Physiology of The University of Chicago

Received for publication September 17, 1942

Whether severe hemorrhage can lead to true shock is still controversial, as the recent literature reveals. If the reduction in blood volume which follows severe hemorrhage fails to produce shock, the theory which attributes the development of traumatic shock to oligemia, resulting from local fluid loss at the site of injury, would be weakened considerably. The work presented here may contribute to the clarification of this problem by demonstrating that shock, with its characteristic manifestations as set forth by Moon, can follow hemorrhage under certain experimental conditions.

Some investigators (Moon; Coonse *et al.*; Mahaffey)[2] have reported that hemodilution invariably follows hemorrhage and have used this observation as the basis for a differentiation between the effects of hemorrhage and shock, emphasizing that, after hemorrhage, hemoconcentration may occur only terminally. More recently, Moon (1) again has stressed that, despite similarities, hemorrhage and shock should be differentiated, clinically and experimentally, lest erroneous conclusions be drawn. Price (2) concurred that in normal anesthetized dogs severe hemorrhage always produces hemodilution and that the outstanding physiologic and pathologic effects of acute hemorrhage are not found in shock produced by other means.

Others (Blalock; Freeman; Davis)[2] have contended that after severe hemorrhage all of the postulated signs of shock, including hemoconcentration, may develop. Blalock (3) and Harkins (4), in their recent reviews, conclude that the differentiation of hemorrhagic from other types of shock is unsound, and that the sequelae of post-hemorrhagic oligemia may include hemoconcentration, if sufficient time is allowed to elapse before death or therapy.

In the light of Starling's theory of fluid exchange in the capillary bed, some hemodilution must necessarily occur after hemorrhage, due to the shift of body water into intravascular circulation as the capillary hydrostatic pressure falls. In well hydrated animals, the early hemodilution following hemorrhage is so great that it can either compensate for extensive blood loss or else (partially or totally) mask subsequent hemoconcentration. It was felt that if the effects of such hemodilution were reduced by deprivation of water, hemoconcentration might be demonstrated to occur after graded bleeding, a procedure by which the fluid reserves of the body are known to be depleted severely (5).

[1] Supported by a grant from The Michael Reese Research Foundation.

[2] All references without index numbers can be found in reviews 3 and 4.

METHODS. Twenty-six healthy, adult, unanesthetized mongrel dogs, weighing from 5.7 to 22 kgm., were used. The animals were starved for 30 hours. Eleven dogs were given water ad libitum. The 15 "dehydrated" dogs were deprived of water for 18 to 30 hours and, in the cooler weather, were exercised for 30 minutes the day before the experiment.

Blood pressures were recorded with the glass capsule manometer (6), which gives a fairly good indication of pulse pressure. Heparin was used as the anticoagulant. The following determinations were performed in duplicate: circulating time by the sodium cyanide method (7); *thiocyanate dilution* by the method of Crandall and Anderson (8); plasma volume by the "direct" dye method (9), using the spectrophotelometer, calibrated syringes and washing out the syringes with blood after dye injections; plasma protein determinations by the micro-Kjehldal method of Ma (10); non-protein nitrogen by direct Nesslerization (11); arterial CO_2 by the manometric method of Van Slyke and Neill; hematocrits with Wintrobe tubes, centrifuged for one hour at 2,500 R.P.M.; hemoglobins with a photoelectric hemoglobinometer; and red cell counts in the usual manner. Blood samples were taken after draining the stagnant blood from the cannulas.

The experimental procedure was as follows: Under local (procaine) anesthesia, the right femoral and right carotid arteries were cannulated and the left femoral and the right external jugular veins were exposed. A control (arterial) blood sample of 15 to 20 cc. was drawn, and sodium thiocyanate solution was injected intravenously. Then, carotid arterial blood pressure and circulating time were recorded. Forty-four minutes after the injection of the thiocyanate, the blue dye was injected. Sixteen minutes later, the first dye sample was drawn from the femoral artery, and, at 4 or 5 minute intervals, 3 or more successive samples were drawn. Hematocrit determinations were made on alternate dye samples. Immediately after the last sample was drawn, the animal was bled a total of 20 to 30 per cent of its estimated blood volume in a 10 minute period. Blood volumes were calculated as $\frac{1}{13}$ of body weight. Carotid blood pressures were recorded during the bleeding periods. Then the arteries were clamped and the animal was placed on the floor, restrained only by a leash. After about thirty minutes the compensation to the hemorrhage was assumed to be complete (12), and the animal was bled an additional 10 to 15 per cent of its estimated blood volume in a 10 minute period. In this and in all successive bleedings if the mean arterial pressure fell below 45 to 50 mm. of Hg, the bleeding was stopped and the animal removed from the board; thirty minutes later, a third bleeding was performed if the animal's condition permitted it.

Circulatory collapse from hemorrhage or trauma was generally characterized by a persistent fall in the blood pressure to less than 70 mm. Hg, by a progressive increase in the circulating time to 20 seconds or more, and by a decrease in the arterial carbon dioxide content to less than 26 volumes per cent. In this investigation, an animal was considered to be in shock only when at least two of these three criteria were present. Generally, the changes in all three were parallel but, not infrequently, one would give more or less normal values when the other two gave a more accurate picture of the animal's critical condition.

After an average period of 35 minutes following the final hemorrhage, the animals were in shock; a 15 cc. sample of blood was drawn for the various determinations and the dye was injected for the second plasma volume determination. All of the animals died some time after the final hemorrhage unless serum or plasma infusions were administered.

RESULTS. The experiments are divided into two main groups, "non-dehydrated" and "dehydrated" animals.

TABLE 1

Average values for all experiments

	NON-DEHYDRATED ANIMALS		DEHYDRATED ANIMALS	
	Hemodiluting	Hemoconcentrating	Hemodiluting	Hemoconcentrating
No. of dogs	9	2	7	8
Wt. (kgm.)	14.7	8.2	10.7	10.2
"Extra-cellular" fluid (cc./kgm.)	256	253	227	227
Plasma volume (cc./kgm.)	36.0	42.0	36.4	38.5
Circulating blood volume (cc./kgm.)	73.6	79.4	74.3	75.3
Total hemorr.				
Cc./kgm	36.0	35.7	32.2	32.0
% of control blood volume	49.0	44.9	43.3	42.5

	TIME OF DETERMINATION							
	Control	Post-hemorr.	Control	Post-hemorr.	Control	Post-hemorr.	Control	Post-hemorr.
Hematocrit	51.6	43.3	50.2	54.6	50.7	45.2	48.9	53.5
Hemoglobin (grams %)	18.1	14.7	17.5	19.3	17.5	15.4	16.2	17.8
Erythrocyte count (10^6/mm.^{3})	7.8	6.4	6.9	7.6	7.1	6.0	6.6	7.5
Mean blood pressure (mm. Hg)	148	58	121	47	126	51	140	48
Circulating time (seconds)	10.4	38.5	14.5	32.0	9.0	42.3	9.6	39.5
Arterial CO_2 (vol. %)	46.4	15.7	39.0	5.1	44.2	17.9	46.6	16.8
NPN (mgm. %)	32	44	47	62	41	59	40	60
Plasma protein (grams %)	5.8	4.7	6.3	5.2	6.1	5.0	6.7	5.9

Hemoconcentration occurred in 2 of the 11 "non-dehydrated" and in 8 of the 15 "dehydrated" animals, the average increase in hematocrit being 4.4 and 4.6, respectively. The average decrease in hematocrit for the 7 dehydrated hemodiluting animals was 5.5, whereas in the 9 non-dehydrated hemodiluting animals it was 8.3. The changes in hemoglobin and red cell counts were in the same direction.

The total blood loss which led to shock differed significantly in the various groups. By total blood loss is meant all measured blood lost in hemorrhages, samples, washing out cannulas, etc. The non-dehydrated hemodiluting dogs withstood an average withdrawal of nearly 50 per cent of their average control blood volume, whereas both groups of dehydrated dogs and the non-dehydrated

hemoconcentrating dogs tolerated an average loss of only 43 per cent of their average initial blood volume.

The changes in total plasma protein concentration revealed that in the dehydrated hemoconcentrating group the decrease in the concentration of total protein, after hemorrhage, was significantly less than in the other groups.

An increase in non-protein nitrogen concentration was observed in every animal except in one of the hemodiluting, non-dehydrated group. The grestest increase (50 per cent) was found in the group of dehydrated hemoconcentrating animals.

Average mean blood pressures, 30 minutes after the last hemorrhage, were at shock levels in all groups. Significantly, the blood pressures were highest in the non-dehydrated, hemodiluting animals, although this group had been subjected to the severest hemorrhage (nearly 50 per cent of the average initial blood volume). Pulse pressures were greatly reduced, indicating decreased cardiac output and depleted circulating volume. The importance of low pulse pressures in the diagnosis of shock has recently been given new emphasis (13).

Arterial blood CO_2 content was decreased to very low levels in the animals of all groups.

Circulating time was markedly increased in every animal.

The general condition of the animals when shock was present appeared to be the same in all four groups. It seemed impossible to differentiate dehydrated from non-dehydrated or hemodiluting from hemoconcentrating dogs. All the animals in shock were listless, not responsive to stimuli, and unable to walk. Severe diarrhea occurred in a number of animals of each group and, often, was bloody. In animals in this condition, withdrawing a 10–15 cc. blood sample frequently induced a terminal circulatory collapse within a few minutes. In several such cases, infusion of 5 per cent dextrose in lactate Ringer's solution, in amounts greater than the total volume of blood lost, was attempted but failed to restore the circulation and death soon occurred.

"Extra-cellular" fluid volumes, as measured by thiocyanate dilution, varied too much in the individual dog to permit correlations to be made. It is interesting to note that, despite great individual variations in both groups, the average value for the dehydrated animals was 227 cc./kgm., whereas the average value for the non-dehydrated animals was 255 cc./kgm., both values at the lower range of the normal limits of 230 to 425 cc. per kgm. (14).

Plasma volume values before hemorrhage in all of the animals fell within usual normal ranges (14). We have confirmed the findings of others (15, 16) that plasma volumes, measured by the blue dye, not only give a good index of the clinical condition of the animal, but also correlate well with the other determinations (v.s.) and thus seem to be a reliable measure of the intravascular plasma volume in shock animals. However, because of the uneven distribution of red cells in the circulation and of the unknown amounts of non-circulating red cells, calculated total red cell volumes and total blood volumes are very inaccurate (2, 17) and, consequently, are quantitatively not significant.

In table 2 a comparison of the average percent change in the plasma volume after hemorrhage with the average percent of the control blood volume which

was withdrawn during the period between the first and the second plasma volume determinations indicates that the animals which hemoconcentrated apparently lost plasma fluid and plasma protein in addition to that removed in the hemorrhage, whereas the animals which hemodiluted apparently drew protein-poor fluid into the circulation, during and after the hemorrhage, and had retained it at the time the second plasma volume was determined. The apparent change in circulating red cell volume, and, consequently, the change in total blood volume are less significant for the reasons mentioned above. The validity and interpretation of the apparent loss of additional red cells by the hemodiluting animals and the relative gain in red cells in the hemoconcentrating animals are not established, as yet.

TABLE 2

Average volumes for experiments in which post-hemorrhage plasma volumes were determined

	NON-DEHYDRATED		DEHYDRATED	
	Hemodiluting	Hemoconcentrating	Hemodiluting	Hemoconcentrating
No. of dogs	5	1	5	5
Hemorr. between control and post-hemorrhage plasma volume determinations				
Cc./kgm.	29.3	36.0	28.8	29.3
% of control blood volume	39.8	39.8	36.8	38.2

	CONTROL	POST HEM.	% DECREASE	CONTROL	POST HEM.	% DECREASE	CONTROL	POST HEM.	% DECREASE	CONTROL	POST HEM.	% DECREASE
Plasma volume (cc./kgm.)	36.0	26.2	27.2	45.1	24.4	46	37.5	26.4	29.5	41.0	23.8	42.6
Circulating blood volume (cc./kgm.)	74.0	46.1	37.6	90.2	55.7	39.3	78.3	51.7	34.0	76.8	47.0	38.8
Total circulating protein (grams/kgm.)	1.9	1.13	40.5	2.57	1.0	61.1	2.43	1.54	37.0	2.59	1.21	54

Pathological findings were variable, as others (13) have reported. Only 11 animals which did not receive infusions will be considered. The gross findings were not consistent in all cases of each group. Generally, the gut was moist, flabby and atonic, and the lumen often contained gross blood. The intestinal mucosa often was engorged and hemorrhagic throughout. The peritoneal and pleural cavities rarely contained increased amounts of free fluid, and, in only one case, was this blood-stained. The lungs, in some dogs, were engorged and moist but, generally, they presented a fairly normal gross appearance. Nothing of significance was found on gross inspection of the other viscera. The outstanding microscopic findings were focal congestion and engorgement of the lungs, marked engorgement and central necrosis in the liver, marked engorgement of the zona fasciculata of the adrenals, and marked engorgement of the intestine and kidney.

DISCUSSION. Hemoconcentration, as measured by hematocrit, red cell count, hemoglobin, etc., has been advanced as a *sine qua non* of shock. Starting from the premise that hemorrhage is followed by hemodilution, Moon (1) has warned investigators not to apply incorrectly to "true" shock conclusions drawn from animals subjected to hemorrhage. Our results differ markedly from his in certain important respects, for by uncomplicated graded hemorrhage we have produced all of the symptoms and most of the important signs which he contends appear only in "true" shock. Moreover, these findings were observed in a number of animals long before death and in others which survived after serum or plasma infusions, and, therefore, cannot be dismissed as pre-terminal phenomena. That hemoconcentration appeared in more animals of the dehydrated group does not affect the conclusion that the oligemia produced by severe hemorrhage, alone, may be sufficient to produce shock.

Adolph (12) has demonstrated that it is unreliable to use the changes in the peripheral blood red cell concentration as estimations of loss or gain of fluid from the circulation, since stored red cells can be shifted rapidly into or out of active circulation. Thus, an apparent hemoconcentration may mean not a loss of plasma but a gain of cells by the circulating blood. However, with T-1824, plasma volumes can be determined fairly accurately and, by a comparison of the observed plasma volume after hemorrhage with the expected plasma volume, calculated by subtracting from the initial plasma volume the amount of plasma removed in hemorrhage, quantitative estimations of fluid shifts can be derived. In 37 anesthetized dogs which hemodiluted after prolonged bleeding, Price (2) reported a significant average gain of fluid. Similar calculations led us to the conclusion that our dogs which hemoconcentrated actually lost fluid from the blood stream in addition to that lost by hemorrhage, whereas the hemodiluting animals tended to retain fluid gained after the hemorrhage. From this it is clear, therefore, that the hemoconcentration which we observed in a number of dogs was due to the loss of additional fluid from the circulation after hemorrhage.

From the total circulating protein before and after hemorrhage (table 2) and the amount of protein removed in bleeding, it can be calculated that the animals which lost fluid actually lost protein from the circulation, too. If allowance is made for this protein loss, the decrease in plasma protein concentration after hemorrhage in the hemoconcentrating animals indicates that initially, like the hemodiluting animals, these animals must have drawn a relatively protein-poor fluid into the circulation. Thus the total fluid ultimately lost, after the animals began to hemoconcentrate, actually must have been greater than the amounts calculated.

These changes in plasma protein concentration also indicate that the dehydrated hemoconcentrating animals could hemodilute less than all the others. Calvin (18) has shown that, in response to moderate hemorrhage, dehydrated dogs can hemodilute less than normal ones. This possibly explains the inability of our dehydrated animals to tolerate as much bleeding as the hydrated animals before going into shock. This, also, may have decreased the masking effect of the earlier hemodilution upon the later outpouring of fluid from the circulation as shock develops and progresses.

One important question remains: why did 2 of the 11 non-dehydrated dogs display hemoconcentration and why did only 8 of the 15 dehydrated dogs display hemoconcentration? This may be part of the broader problem of why some investigators have been able to observe hemoconcentration following hemorrhage and others not. Blalock (3) and Harkins (4) have stressed the possibility that this discrepancy may depend on the time factor, stating that sufficient time must elaspse after hemorrhage before hemoconcentration can occur. But whether hemoconcentration will occur after hemorrhage may depend as much, or even more, on the state of hydration of the animal and on the physiological and pathological variables which affect hydration. A supposedly "normal," non-dehydrated animal actually may be dehydrated and in poorer condition than a healthy young animal that has been dehydrated experimentally for 30 hours. Until more accurate means of determining intra-cellular and extra-cellular water are developed, the evaluation of the factor of hydration in an individual case will be difficult. Our group averages for "extra-cellular" fluids (i.e., thiocyanate dilution) point in that direction, however.

Under the stress of modern warfare, dehydration of combatants may occur and severe hemorrhage may produce shock with hemoconcentration. It would seem to be unwise, therefore, to differentiate hemorrhagic shock from shock produced by other causes. Further, our studies indicate that the appearance of hemoconcentration following hemorrhage is a *signum male ominis*. The clinician who believes that only hemodilution can follow hemorrhage, in the absence of hemodilution, may have a false sense of security in the belief that the patient has not lost much blood. The opposite may actually be the case. In most instances, fortunately, the prevailing clinical picture will give adequate warning of the patient's critical condition.

SUMMARY

1. Typical shock has been produced by graded hemorrhage in 11 non-dehydrated, and in 15 dehydrated, unanesthetized, normal dogs.

2. In 2 of the non-dehydrated (18 per cent) and in 8 of the dehydrated animals (53 per cent), hemoconcentration occurred. Plasma volume and plasma protein determinations, before and after hemorrhage, revealed that the animals which hemoconcentrated, actually lost additional plasma fluid and protein as shock developed.

3. Pathologic changes consisting of gastro-intestinal engorgement and hemorrhage, pulmonary congestion and engorgement, and occasional changes in other viscera were observed in a number of animals.

4. The hemodiluting, non-dehydrated animals tolerated an average total blood loss of 49 per cent as compared to an average total blood loss of 43 per cent tolerated by the other animals.

5. The changes in plasma protein concentration after hemorrhage indicated that the hemoconcentrating dehydrated animals hemodiluted during and after hemorrhage to a lesser degree than the other 3 groups. This relative inability to hemodilute could explain their inability to tolerate as much bleeding

before going into shock and could lessen the masking by earlier hemodilution of he subsequent hemoconcentration as shock develops.

6. It is suggested that the conflicting reports as to the occurrence of hemoconcentration after hemorrhage may be related to the state of hydration of the animals studied.

7. It is concluded that there are no definite grounds for differentiating between hemorrhagic shock and shock from other causes.

REFERENCES

(1) Moon, V. H., D. R. Morgan, M. M. Lieber and D. McGrew. J. A. M. A. **117:** 2024, 1941.
(2) Price, P. B., C. R. Hanlon, W. P. Longmire and W. Metcalf. Bull. Johns Hopkins Hosp. **69:** 327, 1941.
(3) Blalock, A. Principles of surgical care; shock and other problems. C. V. Mosby Company, St. Louis, 1940.
(4) Harkins, H. N. Surgery **9:** 231, 1941.
(5) Levinson, S. O., F. Neuwelt and H. Necheles. J. A. M. A. **114:** 455, 1940.
(6) Anderson, F. F. J. Lab. and Clin. Med. **26:** 1521, 1941.
(7) Olson, W. H., H. Gutmann, S. O. Levinson and H. Necheles. War Med. **1:** 830, 1942.
(8) Crandall, L. A., Jr. and M. X. Anderson. Am. J. Digest. Dis. and Nutrition **1:** 126, 1934.
(9) Gibson, J. G. and W. A. Evans, Jr. J. Clin. Investigation **17:** 153, 1938.
(10) Ma, T. S. and G. Zuazaga. Indust. and Eng. Chem., Analytical Edition **14:** 280 1942.
(11) Folin, O. and W. Denis. J. Biol. Chem. **26:** 473, 1916.
(12) Adolph, E. F., M. J. Gerbasi and M. J. Lapore. This Journal **104:** 502, 1933.
(13) Wiggers, C. J. Physiol. Rev. **22:** 74, 1942.
(14) Gregersen, M. J. and J. D. Stewart. This Journal **125:** 142, 1939.
(15) Gregersen, M. J. Personal communication.
(16) Gibson, J. G., II. Personal communication.
(17) Stead, E. A., Jr. and R. V. Ebert. This Journal **132:** 411, 1941.
(18) Calvin, D. B. J. Lab. and Clin. Med. **26:** 1144, 1941.

THE EFFECT OF NEMBUTAL-ETHER ANESTHESIA UPON BLOOD CONCENTRATION

LEONARD W. JARCHO

From the Department of Physiology, College of Physicians and Surgeons, Columbia University

Received for publication September 19, 1942

The correct interpretation of experimental data obtained from anesthetized animals requires an understanding of the physiological abnormalities induced by the narcotic agents employed. Important dislocation of fluid balances is known to occur under anesthesia. Thus, etherized dogs show hemoconcentration (1, 2, and others) associated with a significant fall in circulating plasma volume (3). Several barbiturates on the other hand produce hemodilution in this animal (4). The cat also shows increased plasma volume under nembutal anesthesia (5), but it differs from the dog in that hemoconcentration does not occur with ether (6). Since it seemed possible that ether might reconcentrate the blood of cats previously diluted by nembutal, the effect of combining the two drugs has been studied.

METHODS. Dogs and cats were used in this investigation. Nembutal (30 to 36 mgm. per kgm.) was injected intravenously or intraperitoneally. Ether was administered by means of a positive pressure respirator, thus assuring adequate oxygenation of the blood. Blood ether determinations (7) made upon several animals showed that anesthetic concentrations of ether were present. Blood was drawn without stasis from the saphenous veins of cats and from the jugular veins of dogs. After the heparinized blood had been centrifuged in Wintrobe hematocrit tubes, the supernatant fluid was used for protein determinations. The protein percentage obtained by the refractometer method was checked in many instances by the falling drop technique. Plasma volume measurements were made with the blue dye, T-1824, according to Gregersen et al. (8). All operations were carried out with strict aseptic precautions.

RESULTS. In 24 experiments on 14 cats anesthetic doses of nembutal produced significant decreases in the hematocrit values (average 26.5 per cent) and in the plasma protein concentrations (average 16 per cent). These changes, which suggest that blood dilution has occurred, are in agreement with the report that nembutal increases the plasma volume in the cat (5).

Since it is difficult to obtain blood from unanesthetized cats without exciting the animals, it seemed necessary to rule out the possibility of emotional hemoconcentration preceding the nembutal dilution. This factor was excluded in three experiments by drawing control samples with the animal under ether anesthesia. Under these conditions the usual nembutal dilution occurred. Conley's (6) experiments as well as the following observations demonstrate that the ether did not affect the blood concentration.

The effect of the nembutal anesthesia followed by the administration of ether was studied in eight cats (10 exps.). After control blood had been taken,

the animals were anesthetized with intravenous injections of nembutal. Three blood samples were obtained at 10-minute intervals, and then ether was administered. Nembutal consistently caused a decrease in the hematocrit readings and in the plasma protein concentrations. These values were essentially unchanged by 40 to 60 minutes of ether inhalation (see fig. 1). Control studies showed that the changes in intrathoracic pressure induced by the respirator were without effect upon the results.

The above experiments were compared with a series in which dogs were used. The intravenous injection of nembutal into 3 normal dogs resulted in a fall in the hematocrit values (average 22.9 per cent) and a decrease in the plasma protein concentrations (average 9.3 per cent). In 11 experiments on 5 normal dogs nembutal anesthesia was followed by the administration of ether. Under these conditions the hemodilution (as measured by hematocrit readings and plasma

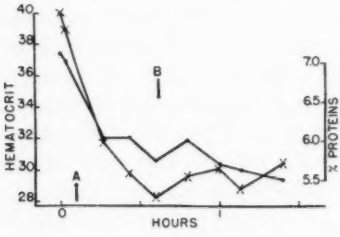


Fig. 1

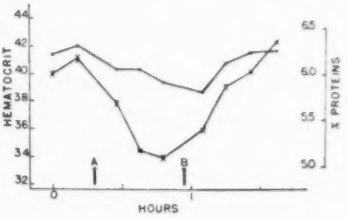


Fig. 2

Fig. 1. The effect of ether inhalation, *B*, on the blood concentration (● = plasma proteins, × = hematocrit) of a normal cat previously anesthetized by means of nembutal, *A*.

Fig. 2. The effect of ether inhalation, *B*, on the blood concentration (● = plasma proteins, × = hematocrit) of a normal dog previously anesthetized by means of nembutal, *A*.

protein concentrations) produced by nembutal injection was succeeded by a marked ether hemoconcentration. Figure 2 shows a typical experiment.

In attempting to explain this discrepancy in the effect of ether one must consider the differences in circulatory response to this anesthetic exhibited by the two species. Thus, it is reported that ether produces vasoconstriction in the hind limbs of the cat (9), whereas in the dog it causes an increased blood flow in the femoral arteries (10). It is possible that femoral vasodilatation may so alter the hemodynamic relations that fluid loss from the circulation is favored. If this is the explanation for ether hemoconcentration in the dog, the same phenomenon should occur in cats which have been deprived of the sympathetic innervation of the hind limbs. However, in 3 cats from which the abdominal sympathetic chains had been removed, ether anesthesia produced no significant changes in hematocrit or plasma protein concentrations. Moreover, ether still failed to produce hemoconcentration in one of these animals after bilateral adrenalectomy.

A further investigation into the difference in the response to ether anesthesia exhibited by cats and dogs was carried out on a number of splenectomized ani-

mals. Seven splenectomized cats responded to nembutal with an average fall of 11.8 per cent in plasma proteins and of 11.2 per cent in hematocrit. In three animals the circulating plasma volume, as determined by the blue dye, T-1824, showed an average increase of 10.1 per cent. The administration of ether 30 minutes after nembutal injection (5 exps.) produced no discernible effect upon the hematocrit, the plasma proteins, or the plasma volume.

In 8 experiments on 7 splenectomized dogs the intravenous injection of nembutal caused an average fall of 4 per cent in plasma proteins and of 3.3 per cent in the hematocrit values. Under these conditions the average increase in the circulating plasma volume was 4 per cent. When ether was administered during the nembutal anesthesia, the plasma protein concentration and the circulating plasma volume returned to the control values, but the hematocrit readings exceeded the control values by about 4.5 per cent.

Discussion. These experiments demonstrate that the injection of nembutal produces hemodilution in normal dogs and cats. In both species the hematocrit values fall to a greater extent than do the plasma protein concentrations. The decrease in protein concentration can be accounted for by augmentation of plasma volume by entering fluid (5), for there is no reason to expect true loss of protein under the conditions of these experiments. The excess fall in hematocrit not accounted for by increased plasma volume indicates egress of cells from the circulating stream, decrease in volume of the erythrocytes, or both. The fact that splenectomy abolishes the difference between hematocrit and protein dilution suggests that segregation of cells in the spleen, dilated by nembutal (see 4), is the important factor. In addition to this activity as erythrocyte reservoir the spleen seems also to be concerned in the movement of the fluid, for splenectomy reduces the amount of protein dilution occurring under nembutal anesthesia. This reduction occurs in both dogs and cats, but is much more marked in the former.

In the dog these changes in blood concentration induced by nembutal are reversed in direction by subsequent etherization: The plasma protein values rise, but the hematocrit is increased even more. The changes are comparable with those resulting from ether anesthesia alone (3). In this case, then, fluid leaves the circulation, while erythrocytes enter it. Splenectomized dogs exhibit the same response to ether, including the excess rise in hematocrit over plasma protein. This would seem to indicate that in the splenectomized dog previously anesthetized with nembutal ether empties some extrasplenic cell reservoir, or else the drug causes a redistribution of erythrocytes in such a manner as to produce an apparent rise in the venous hematocrit. Possible changes in the volume of the individual red cells undetected by hematocrit measurements must once again be mentioned.

The administration of ether to normal and splenectomized cats already under nembutal anesthesia produces no change in plasma volume, plasma protein concentration, or hematocrit values. Ether, therefore, fails to raise the hematocrit in the cat not only normally (5) but also when the animal's spleen has already been dilated (4) by nembutal anesthesia. The failure of the previously un-

anesthetized cat to respond to ether by increase in the hematocrit, in the manner exhibited by the dog, is therefore not attributable to pre-existing maximal contraction of the spleen. The spleens of the two animals apparently react in a different manner to ether. A species difference also exists in the effect of ether on the plasma volume. In dogs, with or without preceding nembutal anesthesia, splenectomized or normal, ether causes hemoconcentration, whereas it fails to change the plasma volume of cats even when the volume has been increased by nembutal. Either the mechanisms which control fluid transfer are not the same in the dog and the cat, or else the action of ether differs in the two species.

SUMMARY

1. In both dogs and cats nembutal anesthesia produces a marked decrease in hematocrit (cats 26.5 per cent, dogs 22.9 per cent) and plasma protein concentration (cats 16 per cent, dogs 9.3 per cent).

2. After splenectomy nembutal anesthesia no longer produces a greater decrease in the hematocrit value than in the plasma protein concentration. The magnitude of both changes is decreased in the splenectomized animal.

3. In dogs anesthetized with nembutal ether raises the previously lowered hematocrit and protein readings above control levels. A similar response occurs in splenectomized dogs.

4. Neither normal nor splenectomized cats anesthetized with nembutal show any evidence of changes in blood concentration in response to ether.

5. The same lack of reaction occurs in cats from which the abdominal sympathetic chains or the chains and both adrenals have been removed.

I should like to thank Dr. Walter S. Root for his constant advice and assistance in these experiments.

REFERENCES

(1) Barbour, H. G. and W. Bourne. This Journal **67:** 399, 1924.
(2) Searles, P. W. J. A. M. A. **113:** 906, 1939.
(3) McAllister, F. F. This Journal **124:** 391, 1938.
(4) Hausner, E., H. E. Essex and F. C. Mann. This Journal **121:** 387, 1938.
(5) Hamlin, E. and M. I. Gregersen. This Journal **125:** 713, 1939.
(6) Conley, C. L. This Journal **132:** 796, 1941.
(7) Ruigh, W. L. Ind. and Engineer. Chem. **14:** 32, 1942.
(8) Gregersen, M. I. et al. This Journal **113:** 54, 1935; **125:** 142, 1939.
(9) Cattell, McK. Arch. Surg. **6:** 41, 1923.
(10) Herrick, J. F., H. E. Essex and E. J. Baldes. This Journal **101:** 213, 1932.

THE MECHANISM OF BILE FLOW INHIBITION UPON DISTENTION OF THE COLON OR STIMULATION OF ITS NERVE SUPPLY

JOHN WARKENTIN, J. H. HUSTON, F. W. PRESTON AND A. C. IVY

From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago

Received for publication September 19, 1942

Goldman and Ivy (1) found that distention of the colon in the dog and monkey caused inhibition of hepatic bile flow. A similar inhibition occurred upon electrical stimulation of the colonic, inferior mesenteric, superior mesenteric, and pelvic nerves. No inhibition was observed by these methods after sectioning of the hepatic nerves. Kuntz and Van Buskirk (2) found that bilateral sectioning of the vagi and splanchnic nerves had no effect on the response which, however, was abolished by application of nicotine to the celiac ganglion. They suggested that the celiac ganglion was a true reflex center. However, in their experiments the lumbar sympathetics were left intact.

The present paper confirms the occurrence of inhibition of bile flow upon colonic stimulation, after section of the vagi and splanchnic nerves, and presents evidence regarding whether the celiac ganglion is a reflex center.

METHODS. We followed the procedure of Goldman and Ivy. The inhibition of hepatic bile flow was studied in 31 dogs under sodium pentothal anesthesia. Ether anesthesia, with morphine and atropine as preanesthetic medication, was also tried, but had no advantage over sodium pentobarbital. The common bile duct was cannulated near the duodenum, and the cystic duct was tied. To distend the colon with water (at 40°C.), the sigmoid colon was tied and a cannula was placed into the cecum or the appendix. Extreme care was exercised at all times to prevent kinking or other obstruction of the bile duct system. During distention the colon was kept outside the abdominal cavity with an intraluminal pressure of 100 to 120 cm. of water for 5 minutes. By means of an electric drop-counter a record of bile flow was kept on a kymographic tracing, which also recorded the blood pressure. For electrical excitation of the colonic nerve or the inferior mesenteric ganglion, a shielded electrode was applied. Stimulations lasted 5 minutes.

After inhibition of bile flow was established, we sectioned the vagi and splanchnics and removed the lumbar chains of sympathetic ganglia. One or more of these lesions were performed in any one dog. Since extensive autonomic nerve lesions at any one operation cause a severe drop in blood pressure, we sectioned the right splanchnics and removed the right lumbar chain under aseptic conditions in a series of animals; such dogs were then studied under anesthesia as described above, from two to three weeks postoperatively.

RESULTS. A. *Dogs Without Any Nervous Lesions. Colon distention.* Out of 12 dogs studied, two gave no bile flow, even after intravenous injection of 3 cc.

of 20 per cent solution of sodium dehydrocholate. Of the 10 "normal" dogs which had a bile flow, colon distention inhibited this flow in 7, no effect being obtained in the remainder. The degree of inhibition of bile flow varied from a slight slowing of flow to a cessation of flow for several minutes.

Electrical excitation of nerves. Out of 10 dogs studied (not the same animals as reported above), one dog had no bile flow at all. Of the other 9 dogs, two showed inhibition of bile flow; the other 7 showed no effect. Because of the irregularity of the response, this method of approach to the study of the reflex activity of the celiac ganglion was discontinued.

B. *Dogs with Nerve Lesions.* *"Acute" nerve lesions.* Bilateral vagotomy in the neck in two dogs did not prevent inhibition of bile flow upon colon distention. Section of the right splanchnics and removal of the right lumbar sympathetic chain in 5 dogs caused such a drop in blood pressure that responses to distention were unsatisfactory and uncertain. Bilateral section of the splanchnics and bilateral removal of the lumbar chains at a single operation caused death from shock in 4 dogs. Because complete severance of the nerves connecting the celiac ganglion with the medulla and spinal cord caused "shock" so frequently, this approach had to be discontinued.

Chronic nerve lesions. In 8 dogs the right splanchnics and right lumbar chains were removed (checked by autopsy) aseptically 3 weeks before the acute experiment. Of 8 dogs, 4 showed inhibition of bile flow upon colon distention. The 4 other dogs had a normal bile flow which could not be inhibited by colon distention.

Since vagotomy, as observed above, does not abolish the inhibitory response, the response observed in these experiments may have occurred *a*, via fibers in the left lumbar chain and splanchnic fibers; *b*, via a true ganglion reflex; *c*, via a ganglionic pseudo-reflex, or *d*, via the blood stream. To rule out possibility *a*, the left lumbar chain and splanchnic fibers were excised.

Two of the preceding dogs, which showed inhibition on colon distention, still showed inhibition of bile flow after the left splanchnics and left lumbar chains were sectioned and excised completely, as confirmed by autopsy. In addition, the vagi were cut in one of these animals.

General observations. Among the conditions which were necessary for a successful inhibitory response, the manner of cannulating the colon was important. Preliminary experiments indicated that the cecal region, including the appendix, most readily gives rise to inhibition of hepatic bile flow. When the colon was distended distal to the cecum, we observed no inhibition of bile flow. Another important factor was the trauma inflicted on the wall of the colon by numerous distentions. If several distentions were necessary to establish the occurrence of inhibition "normally," it was believed that failure to obtain inhibition of bile flow after nerve lesions and further distentions in such an animal could not with certainty be ascribed to the section of the nerves.

The observation was repeatedly made that inhibition of bile flow was even more marked *following* the end of stimulation (distention or nerve excitation) than during the period of stimulation.

DISCUSSION. The results show that distention of the proximal colon of the dog causes inhibition of hepatic bile flow, even after bilateral vagotomy and splanchnic section and bilateral excision of the lumbar sympathetic chains. (Removal of the right lumbar chain with section of the right splanchnic nerves from 2 to 3 weeks previous to the distention of the colon appears to abolish reflex inhibition of hepatic bile flow in some, but does not do so in all dogs.) It follows that the inhibition must be due to either *a*, a true ganglionic reflex; *b*, a ganglionic pseudo-reflex (3); or *c*, a hormone carried by the blood stream. Since it was clearly shown that section of the hepatic nerves abolishes the reflex, a hormone mechanism is ruled out (1). The abolition of the reflex by treatment of the celiac ganglion (2) with nicotine shows that the ganglion is the reflex center for the response, but it does not show whether the reflex is of the nature of a true reflex or a pseudo-reflex. To determine which of the two possible types of reflex activity is concerned, it would be necessary to distend the colon in animals in which the vagi, splanchnic and lumbar sympathetic nerves have been sectioned for a sufficient period of time to permit degeneration of branched visceral sensory axones. It would seem odd, however, for a sensory visceral nerve with a cell body located in the dorsal root ganglion to send one axone branch to the celiac ganglion and another to the proximal colon. Such a concept is more plausible morphologically in the case of Langley and Anderson's (3) observation on the pseudo-reflex of the urinary bladder in which the center is located in the inferior mesenteric ganglion.

CONCLUSIONS

1. Distention of the proximal colon in the dog inhibits hepatic bile flow in about 70 per cent of anesthetized dogs. Inhibition from stimulation of the colonic nerves or the inferior mesenteric ganglion was not so frequently observed in these as in previous experiments.

2. The reflex concerned may be excited after decentralization of the celiac ganglion, by section of the vagi, splanchnic nerves, and excision of the lumbar sympathetics.

3. The evidence strongly indicates that the celiac ganglion is either a true or a pseudo-reflex center for the temporary inhibition of hepatic bile flow which occurs on distention of the proximal colon. The nerve fibers concerned are not located in the vagi, but in either the thoraco-lumbar sympathetics or the prevertebral autonomic system.

REFERENCES

(1) GOLDMAN AND IVY. Annals Surg. **110:** 755, 1939.
(2) KUNTZ AND VAN BUSKIRK. Proc. Soc. Exper. Biol. and Med. **46:** 519. 1941.
(3) LANGLEY AND ANDERSON. J. Physiol. **16:** 410, 1894.

EXCRETION OF THE URINARY ANTIDIURETIC[1] PRINCIPLE IN RENAL HYPERTENSIVE DOGS

D. B. FRANKEL AND G. E. WAKERLIN

From the Department of Physiology, University of Illinois College of Medicine, Chicago

Received for publication September 30, 1942

Studies of the possible rôle of the posterior pituitary in the pathogenesis of experimental renal (Goldblatt) hypertension in the dog and the similar condition of essential hypertension in the human have in the main yielded negative results. Thus attempts to demonstrate the presence of the pressor principle of the posterior pituitary in the blood of hypertensive dogs and in the blood, spinal fluid, and urine of hypertensive humans have been largely unsuccessful. Moreover, total hypophysectomy did not interfere with the development of hypertension following bilateral renal artery constriction in dogs (1, 2), and repeated injections of solution of posterior pituitary did not cause a further increase in the blood pressure of renal hypertensive dogs (3). On the other hand, Griffith et al. (4) reported the production of hypertension in rats by injections of vasopressin and Sattler and Ingram (5) found that injury to the supraopticohypophysial system in dogs with experimental renal hypertension produced significant reductions in blood pressure. The hypothesis that essential hypertension in the human is due to hypersecretion of the pressor substance of the posterior lobe of the pituitary gland was especially championed by Cushing (6).

In order to elucidate this problem further, we have studied the activity of the posterior pituitary in renal hypertensive dogs by assaying their excretion of the urinary antidiuretic principle during normal hydration and during dehydration. Most authorities agree that the urinary antidiuretic substance of the normotensive dog is secreted by the posterior pituitary and that the secretion varies more or less inversely with the state of hydration.

METHODS. Eight dogs were used; four before and after the production of hypertension by the method of Goldblatt (7), two only during normotension, and two only during hypertension. Mean blood pressure readings were obtained by puncture of a femoral artery two or three times a week. Blood urea nitrogen studies, urinalyses, and body weight determinations were made at monthly or bimonthly intervals.

Determinations of the excretion of the urinary antidiuretic principle during normal hydration and during dehydration were made at monthly intervals while the dogs were normotensive and at monthly or bimonthly intervals after hypertension was produced. Four determinations were made during normotension and four during hypertension on each dog except as indicated above. For this purpose one specimen of urine was collected in a metabolism cage during twenty-four hours of normal hydration and a second specimen during a subsequent

[1] This work was aided by a grant from the Graduate School Research Fund of the University of Illinois.

TABLE 1

Excretion of urinary antidiuretic principle in normotensive and renal hypertensive dogs during normal hydration and during dehydration (rat method of Burn (8))

DOG NO.		NORMOTENSION				HYPERTENSION			
		No. of determinations	Time to maximum (urinary) excretion		BP range	No. of determinations	Time to maximum (urinary) excretion		BP range
			Range	Average			Range	Average	
			min.	*min.*	*mm. Hg*		*min.*	*min.*	*mm. Hg*
1	NH	4	85–90	88	122–132	4	85–92	88	160–180
	D	**4**	**119–130**	**123**		**4**	**110–115**	**111**	
2	NH	4	83–86	85	106–118	4	83–90	87	160–170
	D	**4**	**99–110**	**102**		**4**	**107–115**	**111**	
3	NH	4	85–92	88	130–150	4	83–90	87	150–168
	D	**4**	**105–110**	**107**		**4**	**108–116**	**111**	
4	NH	4	84–90	87	115–125	4	88–95	90	148–160
	D	**4**	**105–110**	**107**		**4**	**108–116**	**112**	
5	NH	4	85–89	86	128–136				
	D	**4**	**106–112**	**108**					
6	NH	4	86–88	87	140–150				
	D	**4**	**103–107**	**105**					
7	NH					4	84–88	87	162–184
						4	**103–110**	**106**	
8	NH					4	88–90	89	168–180
	D					**4**	**104–112**	**108**	
Grand average.....				87				88	
				109				**110**	

NH = Normal hydration. D = Dehydration. (Normal hydration figures in regular type; dehydration figures in boldface type.)

Controls

	NO. OF DETERMINATIONS	TIME TO MAXIMUM EXCRETION			NO. OF DETERMINATIONS	TIME TO MAXIMUM EXCRETION	
		Range	Average			Range	Average
		min.	*min.*			*min.*	*min.*
Vasopressin 2 mu/100 gm. of rat	6	140–150	144	Vasopressin 8 mu/100 gm. of rat	4	190–200	195
Vasopressin 4 mu/100 gm. of rat	16	160–180	170	Physiological saline 1.5 cc./100 gm. of rat	11	76–86	82

Mu = milliunit.

forty-eight hour period of water deprivation. After filtration, each urine specimen was dialyzed in a cellophane bag against running tap water for three hours, and concentrated to a volume of 8 to 15 cc. at an absolute pressure of 20 to 30 mm. Hg and 35–38°C. Each concentrate was then assayed for antidiuretic potency by injection into a group of four rats, using the method of Burn (8) which involves the determination of the so-called "time to maximum (urinary) excretion." Control assays were conducted with vasopressin (Pitressin)[2] and with physiological salt solution.

RESULTS. The results are summarized in table 1. The dogs remained in excellent condition throughout the study as shown by their appetites, body weights, and normal urinalyses and blood urea nitrogens.

DISCUSSION. The results demonstrate clearly there was no significant difference in the amount of the antidiuretic principle in the urines of the renal hypertensive dogs, as contrasted with the normotensive animals, either during normal hydration or during dehydration. Dehydration produced a like appearance of the principle in experimental renal hypertension as in normotension. The results consequently do not support, but do not rule out, the possibility that the activity of the posterior lobe of the pituitary is altered in experimental renal hypertension.

CONCLUSIONS

1. The excretion of the urinary antidiuretic principle in dogs during normal hydration and during dehydration was not changed by the production of experimental renal (Goldblatt) hypertension.

2. These results do not support, but do not rule out, the possibility of altered posterior pituitary function in experimental renal hypertension in the dog.

REFERENCES

(1) PAGE, I. H., AND J. E. SWEET. This Journal **120:** 238, 1937.
(2) GOLDBLATT, H., S. BRADEN, J. R. KAHN AND W. A. HOYT. J. Mt. Sinai Hosp. **8:** 579, 1942.
(3) WAKERLIN, G. E. AND W. GAINES. This Journal **130:** 568, 1940.
(4) GRIFFITH, J. Q., JR., H. O. CORBIT, R. B. RUTHERFORD AND M. A. LINDAUER. Am. Heart J. **21:** 77, 1941.
(5) SATTLER, D. G. AND W. R. INGRAM. Endocrinology **29:** 952, 1941.
(6) CUSHING, H. Am. J. Path. **10:** 145, 1934.
(7) GOLDBLATT, H., J. LYNCH, R. F. HANZAL AND W. W. SUMMERVILLE. J. Exper. Med. **59:** 347, 1934.
(8) BURN, J. Quart. J. Pharm. and Pharmacol. **4:** 517, 1931.

[2] Generously supplied by Dr. Oliver Kamm of Parke, Davis and Company, Detroit, Michigan.

SYMPATHETIC AND VAGAL INTERACTION IN EMOTIONAL RESPONSES OF THE HEART RATE

D. D. BOND[1]

From the Department of Physiology in the Harvard Medical School

Received for publication August 24, 1942

This paper reports an attempt to examine more completely than hitherto the activity of the autonomic system, as manifested in changes of cardiac rate, in response to emotional stimulation, and to correlate the data obtained with present knowledge of these systems gathered from more direct examinations.

METHOD. Unanesthetized dogs and cats were used. Heart rates were recorded by a Grass ink-writing galvanometer driven by a resistance-capacity coupled amplifier. The electrodes were of solder. One was placed precordially, the second to the right chest wall, and they were both held in place by an elastic band encircling the chest. When respiration was registered, a small blood-pressure cuff was wrapped around the animal's chest and fixed. Changes of pressure in the cuff were recorded either by a tambour which wrote on a kymograph or by a piezo-electric crystal which was connected to an amplifier and recorded by another pen of the ink-writer.

The animal under observation was placed in a cage, and the leads for the electrocardiogram and respiration were led out the top. The cage was large enough to allow the animal considerable freedom of movement.

After the animal was placed in the cage an interval (30 min. to 2 hrs.) was allowed for a constant slow heart rate to obtain. Great care was needed, especially with dogs, to exclude extraneous noises; and quiet was maintained inside the room. The ink-writer was usually enclosed to minimize the noise of its motor. Stimulation was produced by the noise made by hitting a table top with an iron rod 3 or 4 times in less than 2 sec. or by a pistol shot. The animal was unable to see the observer at any time and in either form of excitation no warning was given.

Observations were made on intact animals and on the same animals after various extirpating operations had been performed. The operations were performed aseptically under ether anesthesia with artificial respiration. A period of a week was allowed to elapse between operations when more than one was performed on a single animal. Observations were made usually not sooner than 24 to 48 hrs. after operation, nor later than 1 month after the first operation. The latter precautions were taken to exclude possible post-operative depression on the one hand, and regrowth on the other. It was soon found that the form of response was remarkably similar in different animals and repeatable in the same animal, so that the observations made on any one subject were few in order to avoid the complication of conditioning.

[1] Fellow of the Rockefeller Foundation.

To obtain accurate graphical representation of the changes in cardiac rate and rhythm, the rate per minute was calculated for each R-R interval in the electrocardiogram and was plotted against the time corresponding to the middle of that interval. The plotting was done on a semi-logarithmic scale in order to show percentile and absolute values.

When it was desirable to show long-range changes in rate, the number of beats per 10 sec. was converted into number of beats per minute and plotted against the time corresponding to the end of the 10 sec. period.

Results. When an unexpected noise was made the animals gave a quick start, took several breaths, or changed position. Dogs often got up if lying down, whereas the cats rarely did.

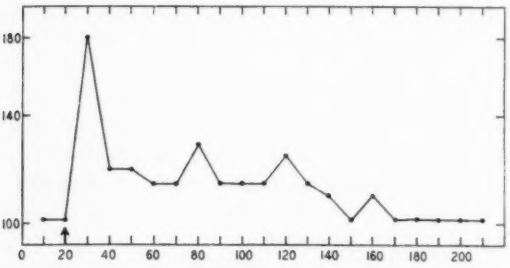


Fig. 1

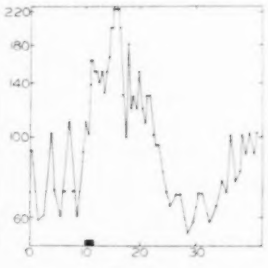


Fig. 2

Fig. 1. A typical response of the heart rate of a dog to unexpected stimulation (at arrow). Ordinates: heart rate per min. calculated from beats per 10 sec. (dots), logarithmic scale. Abscissae: time in seconds.

Fig. 2. The early part of a response of the heart rate of a dog in detail. In this and all following similar figures the rate per minute for each beat has been calculated and plotted (dots) with ordinates: number of beats per minute, logarithmic scale; abscissae, time in seconds; heavy black line, period of stimulation.

A. *Normal animals.* Intact dogs gave a remarkably constant response to a startling stimulation. Variations were small, and when they occurred usually were repeatedly demonstrable for the individual. The response began within one-fifth of a second or less with a loss of the respiratory rhythm of the pulse and an increasing heart rate, which progressed, with interruptions, to a maximum reached in 3 to 4 sec. The rate of individual beats at this peak was 225 per min. or over and was maintained for 4 to 6 sec. At this time the heart rate usually fell toward the control level, sometimes crossing it. Characteristically, this fall was sudden and began with one or more very slow beats, ordinarily lasted about 10 sec., and was succeeded by either a flattening at this level or a significant rise on a slope, more gradual than that of the initial rise which reached a peak, variable in height, at 45 to 60 sec. after the stimulus. From this time onward undulations in the rate, sometimes at quite regular intervals, were common. All evidence of the response was gone usually after 2 to 3 min. but it would occasionally last as long as 7 min. (see figs. 1 and 2).

A not infrequent variation from this picture was the addition of several beats at individual rates up to 300 per min. in the first 2 sec. after stimulation. These beats were followed by a slight fall and then a gradual rise along the curve just described

In normal dogs the resting heart rates varied from 100 down to approximately 60 per min. A marked variation with respiration was usually present when the rate of the pulse and of the respiration was slow. Within the limits just mentioned the resting level of the cardiac rate seemed to have very little influence on the height of the initial response. However, the fall secondary to the initial rise was usually more noticeable, but quantitatively no greater, in the animals with a higher rate and no variation with respiration during the control period, than in the others (cf. figs. 2 and 5). When a variation with respiration was present in the control period, the rate of the slowest beats during the fall usually corresponded closely to that of the slowest beats of the control period (fig. 2). The determination of the cardiac rhythm by the respiration, however, is not dependent upon the rate of the respiration and of the pulse alone (p. 477). Frequently, a change in respiratory cardiac rhythm was the first evidence of response and the rhythm often returned to the degree of influence present during the control period after the cardiac rate had reached its control level. Exceptionally, the sinus arrhythmia could be maintained throughout the response with the rates of individual beats being as high as 200 per min. (fig. 3).

What has been described for the reaction of dogs can, in large measure, be said for cats. Quantitatively, the height of response was similar in the two species, and members of each group showed a few very rapid beats at the beginning of the reaction. Variability was more marked with cats and was seen not only in the group as a whole, but in the same individual from time to time. A frequent deviation from the irregular response shown by dogs was a smoother curve, quite similar to that of dogs with the vagi cut (see p. 471, and fig. 4). The initial increase in rate following stimulation often terminated in 5 sec. to be followed successively by a slight fall and a peak, higher than the first, occurring at 15 to 30 sec. The fall in rate, secondary to the initial rise, so typical of the dogs, was not so profound in cats, and in the series never crossed the control level. Whereas the reaction of cats could be indistinguishable from that of dogs in the first 10 sec., the later part of the response often showed quite variable changes in rate that could go to very high levels. These second rises in rate tended to come earlier than in dogs; i.e., in 20 to 40 sec. rather than in 45 to 60 sec.

The first 25 sec. of an unusual response in a normal cat is shown in figure 3. Immediately after the stimulus the record was spoiled by movement artifact. However, the mechanism of slowing by increasingly longer intervals between beats during expiration, with the maintenance of very short beats during inspiration, is well shown.

B. *The influence of adrenaline.* Adrenaline was excluded from the circulation by removal of one adrenal and denervation of the other. When this was done, the only change detectable in the response of either the dog or the cat was an abolition of, or a marked decrease in, the magnitude of the late accelerations.

The heart of one cat was denervated to act as an indicator for the appearance of adrenaline. This cat was startled repeatedly. The maximal acceleration obtained was a gradual increase of 30 beats per min., which began at 14 sec. after stimulation and was maintained on a constant plateau for 4 min. In no instance was there any evidence of an increase in rate occurring before 12 sec. from the stimulus.

C. *Accelerator activity.* Dogs with the vagi cut in the neck and adrenaline excluded, were subjected to experiment 24 to 48 hrs. after operation. It was necessary in these animals to punch the vocal cords to permit adequate respiration. The resting level of the pulse in such animals was 120 to 130 beats per min., and the rhythm was quite regular.

The response to a stimulus was prompt, the heart rate rising perceptibly within 1 to 2 sec.; i.e., within the first 1 to 3 beats. The rate rose on a smooth, steep

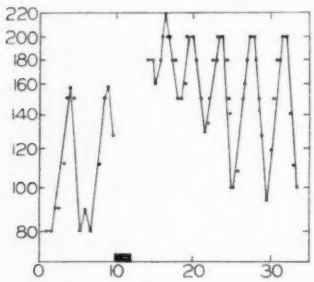


Fig. 3

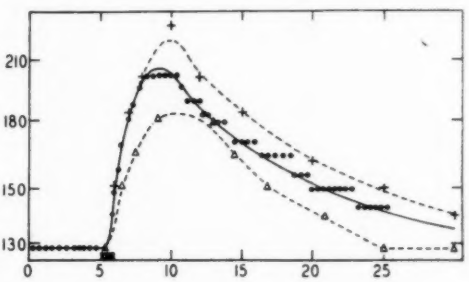


Fig. 4

Fig. 3. The early part of an unusual response in a normal cat. Note the maintenance of respiratory arrhythmia and the slowing effected by increasingly longer beats in expiration.

Fig. 4. Accelerator response. Three experiments from two dogs with vagi cut and adrenaline excluded. In the middle curve each beat is represented by a dot. The steppage effect is due mainly to an artifact introduced in the measurement. Only key points are plotted in the other two curves. Note total lack of respiratory arrhythmia.

slope to reach, in 3 to 5 sec., a maximum which was maintained for 3 to 4 sec. The rate then fell on almost a straight line, returning to the initial level in 20 to 25 sec. from the time of stimulation. Figure 4 shows three experiments on two different animals. Figure 5 show one of the curves superimposed upon the pattern obtained in the same animal before any operation.

No similar group of experiments was done on cats with the vagi severed, because the resting heart in the animals so prepared was too rapid (over 200 per min.) to allow an adequate response.

D. *Vagal activity.* Dogs were deprived of their cardiac sympathetic supply by removal of the stellate ganglia and the thoracic sympathetic chains as far down as the 6th rib. Adrenaline was excluded from the reaction.

The most striking changes in the records of these animals after the operation were the somewhat slower basal rate (48 to 60 beats per min.) and the greatly increased prominence of the respiratory influence on the cardiac rhythm.

These animals also reacted to the startle very promptly—usually a change could be detected within the first beat. In the more usual response the pulse rate did not rise above the rate of a denervated heart; i.e., 100 to 110 beats per min. This rate usually was only slightly higher than that of the fastest beats of the control period (fig. 6). By far the most striking feature of these animals was a change in cardiac rhythm. There was usually a complete loss of the slower beats occurring in late expiration, and it was this loss that in great measure was responsible for the increase in rate.

In a more exceptional response, typical only of a few individuals, the first sign of reaction was 4 to 5 beats of very high rate (200 to 350 per min.) occurring immediately (0.2 sec.) after the startle. These fast beats were then followed by

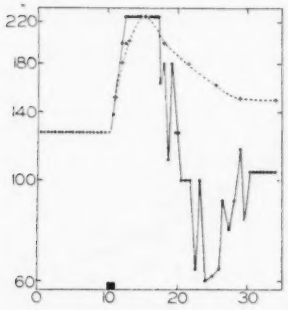


Fig. 5

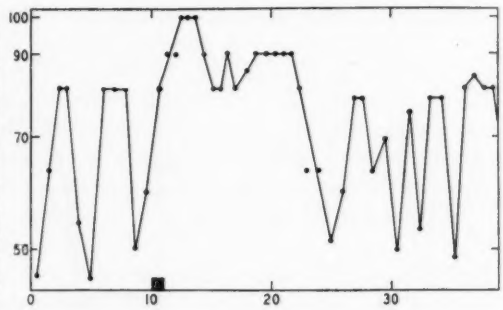


Fig. 6

Fig. 5. Rôle of vagi in secondary slowing. Dots and solid line represent response of a normal dog. Crosses and dash line show the accelerator response in the same dog after severance of the vagi. Note that the control period showed the same pulse rate in both instances and no respiratory arrhythmia.

Fig. 6. Inhibition of the vagus. The response of the dog after removal of the sympathetic accelerators and carotid sinuses bilaterally. Note pronounced respiratory arrhythmia in control period. This response is similar to that of a dog with sympathetic accelerators out but with carotid sinuses not removed. Each beat is plotted.

the course of the response just described. The long intervals occasionally observed early in the course of the response were not seen, however, if these rapid beats were not present (fig. 7).

The response lasted 8 to 15 sec. and showed a very abrupt end, usually being initiated by one very long interval between beats. This interval was sometimes succeeded by similar long intervals of somewhat shorter duration leading to a resumption of normal rate and rhythm. At other times there was a more gradual decline of the response to normal levels, after which normal rhythm returned. In one dog, despite the maintenance of the threatening stimulus for 16 sec., the heart rate began to decline in 12 sec. and normal rhythm was resumed in 25.

In cats with the sympathetic supply to the heart extirpated the response in general shape and time course was quite similar to that of dogs. Fewer irregularities occurred in the curve, however, and, also, several animals on occasion

showed increases of rate well above that of the denervated heart; i.e., up to 150 to 160 beats per min. for 3 to 5 sec. When these animals were presented to a barking dog their heart rates rose to very high levels, 200 or over, despite the inactivation of the adrenals. This rise was prompt, showed a quick decline, and was of such magnitude that it is very unlikely that sympathin could have been the responsible agent (Partington, 1936).

E. *The influence of the carotid sinus.* The carotid sinus was resected bilaterally after the method of Heymans (1933). When this was done in an otherwise normal dog the resting level of the pulse rose from 60 to 100 beats per min., and the rhythm was entirely regular. The response to the unexpected noise was now a smooth curve much like that seen in the group of animals having only the accelerators present (fig. 4). Instead of the profound secondary fall from 150

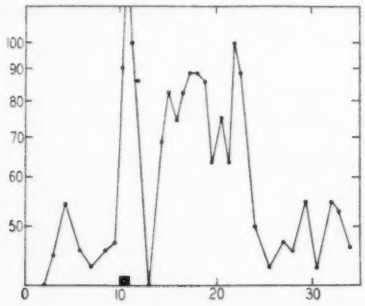


Fig. 7

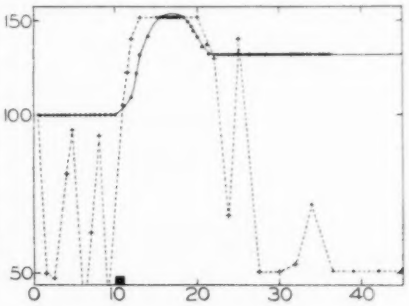


Fig. 8

Fig. 7. Several fast beats of vagal origin seen in a dog with vagi intact but with sympathetic accelerators removed and adrenaline excluded. The beats went off the chart to a rate of 180 per min. Note the profound fall secondary to these fast beats and the sudden termination of the response. Each beat is plotted.

Fig. 8. The effect of removal of the carotid sinuses when the sympathetic accelerators are intact. Crosses and dash line show pattern of normal response (key points only). Dots and solid line show response in same dog after removal of carotid sinuses (all beats).

to 54 beats per min., seen in the control observations on this animal, the heart rate stayed well above the resting level for several minutes and showed no irregularities. The very long duration of this response is attributed in part to the action of adrenaline. It is interesting that the height of the response was identical with that seen in the control observation despite the difference in resting pulse levels (fig. 8).

When the carotid sinus was removed from an animal previously deprived of its sympathetic supply to the heart and with its adrenals excluded, no such dramatic change appeared. The respiratory cycle of the cardiac rhythm was most pronounced, and the duration and shape of the response was identical with that of an animal with the carotid sinuses present (fig. 6).

F. *Influence of respiration.* When either a cat or a dog is startled the first respiratory change is 3 to 5 deep, rapid breaths. These may be followed by

either a continuance of the deep breathing or a period of apnea. Figure 9 illustrates the respiratory change of the latter type in a dog deprived of its sympathetic cardiac supply in response to a very slight noise. It would seem from this record that the corresponding increase in cardiac rate is due to the loss of the slow beats normally occurring in expiration, as no single interval between beats is shorter than those of the control period. This, then, would indicate that the heart was not truly accelerated but that the change in rhythm necessitated by the change in respiration was the sole cause of the faster pulse. On the other hand, a marked deceleration of the heart occurred in an intact cat subsequent to an initial high rise, in the second 10 sec. period following the stimulus. This period of slowing corresponded exactly to a period of apnea. When respiration was again resumed the pulse rate immediately increased.

If the respiratory rhythm, imposed upon the cardiac rhythm, is maintained throughout the response, it plays a very important rôle in shaping the pattern (p. 470 and fig. 3).

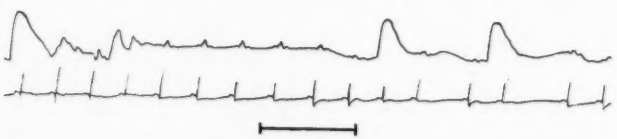


Fig. 9. Cardiac acceleration due to apnea seen in the same dog as in figure 6. Top tracing shows respiration (inspiration up); bottom record, electrocardiogram. The normal rate and rhythm of the heart and respiration are resumed at the right of the record. The acceleration of the heart during the period of apnea has been accomplished solely by the dropping of long intervals. Heavy black line indicates 1 sec.

That the interrelationship of cardiac and respiratory rhythms is complex is shown by the following observations. A dog, deprived of its sympathetics to the heart and its carotid sinus and depressor nerves, showed periods of panting that left the heart rate unaltered at 114 per min. Also, a normal dog which after a startle showed a rise in pulse rate followed by a profound fall to 54 per min., failed to exhibit any sinus arrhythmia despite the maintenance of a uniform respiration of 18 per min. at this time (see normal curve in fig. 8). On the other hand, long intervals between beats, interrupting the course or bringing to sudden termination the faster rate in intact animals and also in those with only the vagus remaining, coincide with the expiratory phase of a deep respiration.

Discussion. From the foregoing data it is concluded that the sympathetic accelerators to the heart are largely responsible for the magnitude of the cardiac acceleration subsequent to brief unexpected stimulation (cf. figs. 4 and 6). Using a technique of stimulation similar to the one presented in this paper, Beebe-Center and Stevens (1937, 1938) have described the reaction of the heart rate of intact cats and a gun-shy dog. They noted the rapidity with which the response appeared and the presence of occasional decelerating vagal activity in its course. The major part of the cardiac acceleration, however, they attributed to inhibition of vagal influence and felt that the sympathetics played only a minor

rôle. They performed no extirpations. Likewise, Whitehorn, Kaufman and Thomas (1935) and Bazett (1941) state that the sympathetic system is too slow in its action to account for rapid changes in rate. In contrast to these statements, the observations described on p. 471 and in figure 4 indicate that the sympathetics are capable of effecting changes easily detectable within 0.5 sec. in many instances. The positive accelerations shown by the progressing decrease in the interval between beats stop in these experiments in from 3 to 7 sec. This time, then, forms a limit within which it is probable that the heightened discharge of the sympathetic fibres ceases.

Rosenblueth and Simeone (1934) have described curves obtained from direct stimulation (for a 10 sec. period at a frequency of 8 per sec.) of the accelerators to the cat heart, which was isolated from the central nervous system. The shapes of the curves are identical with those shown in figure 4. Likewise, Morison (1935) has published curves of similar shape for the reflex speeding of the heart when the vagi are cut. However, the time parameters are at variance in that the curves obtained in response to unexpected stimulation are very much steeper in their initial ascent and return to the resting level more promptly than do the curves obtained from direct or reflex stimulation. Because the shape of the curves obtained from the dogs coincides so well with those obtained by these authors in cats, it is reasonable to infer a similarity of process in the two species.

The assumption that the response of the accelerators after an animal is startled is a brief burst of impulses of high frequency (20 to 30 per sec., see Rosenblueth, 1932) lasting less than 3 to 7 sec., would account for the discrepancy in the time of ascent to high rates between the curves reported here and those described by these other authors. Figure 4 indicates a grading of response which could be explained on the basis of change in either frequency or duration of accelerator discharge; it is similar to a figure reported by Morison in which high frequency of afferent stimulation evoked larger and more lasting reflex acceleration than did low.

The fact that the descent of the curves in figure 4 is steeper than that established by Rosenblueth and Simeone may be due to the carotid sinuses. Pitts, Larrabee and Bronk (1941) have shown, by direct recording, that stimulation of the carotid sinus nerves inhibits the discharge of accelerator impulses. It is interesting, however, that in these experiments by far the greatest factor causing slowing was the vagi, and if they were cut no slowing beyond the initial resting level took place.

Rosenblueth and Simeone have shown that the vagus and accelerators work independently if the effects of either are gauged by the percentile change in heart rate effected by direct stimulation at known frequencies. Likewise, it can be said that in the response to the startling stimulus, the vagus, in the main, acts upon the background offered by the change in rate effected by the accelerators and that its activity is, in part, determined by that background.

This mechanism is well illustrated by the observation that the profound fall in cardiac rate subsequent to an initial rise, more pronounced in dogs (fig. 5), was never seen either in animals deprived of the sympathetics or in those with vagi

cut. It seems probable, therefore, that this slowing is dependent upon the integrity of the vagi, and also upon the attainment of a critical level, either of pulse rate or of blood pressure, which is responsible for tripping a depressor mechanism which acts primarily through the vagi. That the carotid sinus is the initiating factor is implied by the experiment described on page 473 (fig. 8). It is noteworthy that this slowing of the pulse occurs at the time when the speeding of the denervated heart by adrenaline begins, and reaches its maximum: i.e., in 10 to 20 sec. Although no quantitative analysis was attempted the point was clear that those intact animals that responded by a high and sustained rise in pulse rate showed the most pronounced subsequent slowing. Confirmatory evidence may also be derived from the observation that when the respiratory rhythm of the heart mediated through the vagi, was kept throughout the response, it became much more marked when the pulse rate rose to high levels (fig. 3, and see below).

However, there is a degree of reciprocal innervation shown by the vagi that is not covered by what has just been said. There seems to be some accelerating mechanism in the vagus, common to both cats and dogs, that is responsible for the few very rapid beats seen in the first 1 or 2 sec. after stimulation (page 472 and fig. 7). Some of these beats have an ectopic origin as the electrocardiogram shows inverted P or QRS waves. Not all of them fall into this category, however, and because they are few, come so promptly, and are at such a fast rate,it would be an error to classify them as being due to the vagal accelerators described by Jourdan and Nowak (1934).

In dogs deprived of the accelerators and with the effect of adrenaline excluded there is a speeding of the heart upon stimulation which, except for the few fast beats just described, never exceeds the rate of the denervated heart, and, therefore, can be explained solely by the inhibition of vagal tone. This conclusion is in agreement with that of Bouckaert and Heymans (1936), but it is in disagreement with the statement of Brouha, Cannon and Dill (1936) that there is a high degree of cardiac acceleration following slight emotional stimulation in dogs deprived of the sympathetic chains from the stellate to the sacral ganglia.

The inhibition of vagal tone just referred to allows full play to the sympathetic accelerators, but accounts for very little of the acceleration seen in the intact animal.

Cats show little secondary fall in pulse rate in comparison to dogs, and intact animals often yield curves that closely approach those of pure sympathetic activity. This species predominance of the sympathetic is in accord with the report of Bender (1938) and with experience of others working on totally sympathectomized cats and dogs.

That there are important cardio-accelerator fibres in the vagi of cats is shown by the evidence given on p. 472. The data presented throw no light on their nature or the mode of their activity. The time course of their effects, however, coincides with that of both accelerator action and vagal inhibition already described.

Respiratory influences, mediated by the vagi, are of importance in the control of rate and rhythm and are one of the most important sources of decelerating

vagal action. Anrep, Pascual and Rössler (1936), using the innervated heart-lung preparation, have shown that the respiratory cardiac rhythm is of extremely complex origin having many central and reflex components. Generally speaking, those influences which raise vagal tone tend to increase the prominence of the respiratory rhythm, while influences which lower vagal tone decrease it. Anrep et al. thus account for the lack of respiratory arrhythmia in a dog without depressor nerves and carotid sinuses as due to a lowering of vagal tone. This is, of course, correct if it is kept in mind that the question is one not only of the absolute vagal tone but of that value relative to the tone of the accelerators. There may be no detectable respiratory arrythmia in a dog deprived only of its carotid sinuses, figure 8, but after removal of the sympathetics that arrhythmia becomes even more pronounced than in the normal (fig. 6).

Anrep and his collaborators have shown that it is mainly the inhibition of vagal tone in early inspiration that accounts for speeding the heart at that time. They state, further, that large doses of morphine prevent respiratory arrhythmia by greatly strengthening vagal tone, so that a uniform, very slow pulse results. This is analogous to the slowing of the pulse without arrhythmia that occasionally takes place in the period of secondary fall when the respiration is uniform and slow.

Apnea alone may be responsible for either a slowing or a speeding of the heart rate, depending in part upon the rate at the time. Because apnea, presumably secondary to hyperventilation, may occur at the end of the 10 sec. period following stimulation, it may account for some of the slowing seen in this period. This is particularly true in cats in which the carotid sinus mechanism is not so prominent as in dogs. In dogs, however, the carotid sinus may be very largely responsible for the cardiac slowing and the apnea.

The secondary rises in heart rate occurring in dogs after 45 sec. and in cats usually after 30 sec. are attributed to a release of the depressing mechanism, and to the influence of adrenaline which at this time is well established. The distinct impression is gained that adrenaline plays a more prominent rôle in cats than in dogs, as cats are more likely to give high peaks at this time. Despite marked rises in cardiac rate, especially in cats, there may be a return to normal in 40 to 50 sec., which is not interrupted by secondary accelerations. This raises the question as to whether adrenaline was secreted in any significant amounts in spite of marked sympathetic activity of the cardio-accelerators. Although the same kind of response was occasionally seen in dogs, it was more rare probably because the carotid sinus is more active in this species, as is shown by late rises of small degree despite the exclusion of the adrenals. The undulations that often take place after 60 sec. are interpreted as being due to overswinging of the pressor receptors.

SUMMARY

Changes in heart rate of unanesthetized dogs and cats, startled by a short, unexpected noise, were recorded electrically. The cardiac responses from the animals when normal were compared to the responses from the same animals after various nerves had been cut.

Intact dogs and cats yielded a complex pattern of a sudden, high rise in heart rate, beginning immediately after the startle. This was successively followed by a sharp fall, more pronounced in dogs, a second rise of variable height, and thereafter, several undulations in rate until a termination of the response in 2–3 min. (p. 469 and figs. 1, 2, 5).

Adrenaline plays a more prominent rôle in cats than in dogs; but its action in either species appears only after 12 sec.

Dogs in which the vagi and depressors were cut and adrenaline excluded, showed pure accelerator activity. The response was similar to that of the normal in promptness and magnitude, but was simpler with no secondary fall or further undulations (p. 471 and figs. 4 and 5).

In dogs and cats with the sympathetic cardio-accelerators removed and with adrenaline excluded, startle was promptly followed by inhibition of vagal tone (figs. 6 and 7). Cats in addition showed an acceleration that was greater than could be accounted for by loss of vagal tonic influence alone (p. 472 and p. 473). Unless there were a few rapid beats of vagal origin occurring in certain individuals immediately after the stimulus, no fall in rate subsequent to the initial rise was seen. If these rapid beats were present they were commonly followed by one very slow beat only. This seems to indicate that usually the increase of heart rate did not raise arterial pressure to a degree sufficient to trip a depressor mechanism acting primarily through the vagus. Evidence is presented that the carotid sinus is involved (p. 473 and figs. 6 and 8).

The effect of respiration on cardiac rhythm is complex and may greatly affect the pattern of response (fig. 3). Apnea may cause a speeding of the heart (fig. 9) or may be accompanied by a slowing (p. 474).

The discussion deals with the quick activity of the sympathetics, the rôle of the vagus, and with the relation of the responses reported here to those obtained by others from direct and reflex cardiac acceleration.

I wish to thank Dr. Walter B. Cannon for much assistance in this work and for his many constructive suggestions.

REFERENCES

ANREP, G. V., W. PASCUAL AND R. RÖSSLER. Proc. Roy. Soc. **B119:** 191, 218, 1936.
BAZETT, H. C. McLeod's Physiology in modern medicine, St. Louis, 1941.
BEEBE-CENTER, J. G. AND S. S. STEVENS. J. Exper. Psychol. **21:** 72, 1937. Ibid. **23:** 239, 1938.
BENDER, M. B. Proc. Soc. Exper. Biol. Med. **39:** 62, 1938.
BOUCKAERT, J. J. AND C. HEYMANS. J. Physiol. **89:** 4P, 1936.
BROUHA, L., W. B. CANNON AND D. B. DILL. Ibid. **87:** 345, 1936.
CANNON, W. B., J. T. LEWIS AND S. W. BRITTON. This Journal **77:** 326, 1926.
HEYMANS, C., J. J. BOUCKAERT AND P. REGNIERS. Le Sinus Carotidien. Paris, 1933.
JOURDAN, F. AND S. J. G. NOVAK. C. R. Soc. Biol., Paris **117:** 234, 1934.
PARTINGTON, P. P. This Journal **117:** 55, 1936.
PITTS, R. F., M. G. LARRABEE AND D. W. BRONK. Ibid. **134:** 359, 1941.
ROSENBLUETH, A. Ibid. **102:** 12, 1932.
ROSENBLUETH, A. AND F. A. SIMEONE. Ibid. **110:** 399, 1934.
WHITEHORN, J. C., M. R. KAUFMAN AND J. M. THOMAS. Arch. Neurol. and Psychiat. **33:** 712, 1935.

EFFECT OF SEX HORMONES ON THE ERYTHROCYTE NUMBER IN THE BLOOD OF THE DOMESTIC FOWL[1]

ELSIE TABER, DAVID E. DAVIS AND L. V. DOMM

From the Whitman Laboratory of Experimental Zoology, The University of Chicago

Received for publication August 27, 1942

A sex difference in the number of erythrocytes in the blood of the domestic fowl was first recorded by Blacher (1, 2), and has since been confirmed by several workers (6, 11, 13, 14). Similar differences have been reported for the dove and pigeon (17) and for a number of wild American birds (16). In immature fowls Chaudhuri (6) reported that the erythrocyte number was intermediate between that found in adult males and females. Juhn and Domm (13) also observed a similarity in the number of red blood cells in immature males and females up to the sixth month after hatching, at which time the number began to increase in males, although the juvenile level was retained by females. This difference was definitely established when the fowls reached sexual maturity at or before nine months of age.

The belief that this difference is due to hormone control has been strengthened by the lowered red cell count found in castrated fowls (1, 2, 12), and by the higher erythrocyte number in sinistrally ovariectomized poulards which have developed ovotestes, and in bilaterally ovariectomized poulards with testis grafts (12).

Recently it has been reported that various estrogens injected into dogs, monkeys, and rats caused a lowered red blood cell count while injections of testosterone propionate effected an increased number (5, 7, 19, 20, 21). The latter was also found to be true when hypogonadic men were treated with testosterone propionate (15).

A study of the effects of androgens and estrogens on the erythrocyte numbers in normal roosters, capons and poulards was undertaken in this laboratory in connection with other observations on the effects of these hormones on secondary sexual characters and behavior. The investigation was extended to include a group of young fowls treated with pregnant mare serum gonadotropin and two groups of intersexual males. A preliminary report of the results has already been published (18).

MATERIALS AND METHODS. Blood was obtained from the wing vein, and diluted with 1.5 per cent solution of sodium citrate in 0.85 per cent sodium chloride.

[1] This investigation was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of The University of Chicago. Grateful acknowledgment is made to Dr. Erwin Schwenk of the Schering Corporation for the testosterone propionate (Oreton) and alpha-estradiol benzoate (Progynon-B), to Dr. J. A. Morrell of E. R. Squibb and Sons for the diethylstilbestrol and for the non-crystalline estrogenic substance (Amniotin), to Dr. Edward A. Doisy of the St. Louis University School of Medicine and Dr. Oliver Kamm of Parke, Davis and Company for the theelin, and to Dr. George F. Cartland of the Upjohn Company for the pregnant mare serum (Gonadogen) used in these experiments.

All counts were made using an improved hemacytometer with a double Neubauer counting chamber.

All the birds used in these experiments were of the single comb light brown Leghorn variety. Uninjected males, females, capons and sinistrally and bilaterally ovariectomized poulards were used as controls. With the exception of the poulards, which were three years old, all adult birds used were one year old.

Six capons, three bilaterally ovariectomized poulards, and three sinistrally ovariectomized poulards received daily intramuscular injections of testosterone propionate. In a similar manner, eleven capons, three bilaterally ovariectomized poulards and two adult males, received alpha-estradiol benzoate or diethylstilbestrol. The amount of hormone given in each case and the duration of the experiment are recorded in tables 2 and 4.

The results obtained from estrogen and androgen injections suggested that an examination be made of immature chicks injected with a gonadotropin. Consequently counts were made on six males and six females which had received daily injections of pregnant mare serum gonadotropin (table 3).

TABLE 1

Erythrocyte counts in uninjected birds

SEX	NO. OF BIRDS	AVERAGE RED CELL COUNT*	RANGE	STANDARD DEVIATION
Male	18	3.60	2.88–4.27	0.361
Female	18	2.70	2.14–3.15	0.312
Capon	18	2.51	2.07–3.10	0.288
Sinistral poulard	12	2.78	2.41–3.34	0.278
Bilateral poulard	3	2.59	2.46–2.71	0.102

* Expressed in millions per cubic millimeter in all tables.

Counts were also made on two series of intersexual males resulting from the injection of various estrogens during early embryonic life. The first group included thirty-six birds about two years old, while the second group included seventeen birds varying in age from 3 to 5 months. The kinds of hormones used, their concentration and the amounts given are indicated in table 5.

Erythrocyte counts made on three intersexual males and six uninjected poulards during the last stages of molt were compared with those made on the same birds the previous summer prior to the molt.

RESULTS. 1. *Normal males, females, capons and poulards.* The average erythrocyte counts for normal males, females, capons, and poulards are recorded in table 1. In the male the average number of red blood cells per cubic millimeter (3,600,000) is approximately a million higher than in the female (2,700,000). The average erythrocyte numbers of the capon and the bilaterally ovariectomized poulard are below, while that of the sinistrally ovariectomized poulard is above the normal female level.

Assuming sex hormones are the controlling factor in erythrocyte production, these results may be interpreted as follows: The development of an ovotestis in

sinistrally ovariectomized poulards is responsible for the production of androgen causing a rise in blood count. In both capons and bilaterally ovariectomized poulards the erythrocyte picture is the same due to the complete absence of gonads, and therefore sex hormones, in each group. The slightly higher count found in normal females is probably due to the production of a small amount of androgen by the medullary component of the left ovary.

From these observations it would appear that androgens stimulate the cells producing red corpuscles, and that a lack of such hormones causes a decrease in their production, but whether the low count in the female is due to the absence of an adequate amount of androgen, to the neutralizing effect of estrogens, or to a direct inhibitory effect of the female hormone cannot be determined from

TABLE 2

Effect of testosterone propionate on erythrocyte number

BIRD NO.	SEX*	MGM. DAILY	RED CELLS PER CU. MM.		
			11th day	24th day	45 days after injections
1**	C	1.00	3.23†	3.35	2.28‡
2	C	1.25	2.91	3.20	2.41
3**	C	2.00	2.68†	2.85	2.66‡
4	C	2.50	2.06	3.21	
5**	C	3.00	3.30†	3.83	3.16‡
6	C	3.75	2.85	3.37	
7	SP	1.25	3.31	3.67	2.52
8	SP	2.50	3.88	4.37	2.81
9	SP	3.75	2.90	3.27	2.86
10	BP	1.25	2.90	3.40	
11	BP	2.50	2.95	3.64	
12	BP	3.75	2.93	2.97	

* C., capon; SP sinistrally, and BP bilaterally, ovariectomized poulard.

** Counts made on 1, 3, and 5 before injections began were 2,400,000, 2,080,000, and 2,170,000 respectively.

† Counts made on 15th day of injection

‡ Counts made 21 days after injections ceased.

these observations. To shed some light on this problem we injected various sex hormones into normal and operated fowls.

2. *Testosterone injected capons and poulards.* The capons and poulards receiving daily doses of testosterone showed an unmistakable increase in the number of red blood corpuscles after 11 days of injection, and this increase was even more noticeable after twenty-four days (see table 2). In every case, the count made twenty-four days after injections were begun was equal to or approaching that of a normal male. Counts made twenty-one days after cessation of injections had fallen appreciably and were approaching the normal level; while counts made forty-five days after injections had ceased had fallen to the level of uninjected capons and poulards.

There appeared little correlation between the amount of testosterone received and the increment of red cells and no attempt was made to find the threshold or

maximum effect doses, although in both the sinistrally and bilaterally ovariectomized poulards a daily dose of 2.5 mgm. seemed to evoke the greatest increment. Further experiments are necessary to prove whether this is approaching the maximum response, or whether the results are merely coincidental. The latter would appear to be the case since there was no correlation between size of dose and blood count in the six capons.

Since counts in all testosterone-injected birds were higher than in normal ones, it might be expected that gonadotropins injected into immature birds would bring about a rise in the juvenile number of corpuscles due to the androgens precociously produced by the stimulated gonads.

3. *Immature males and females injected with pregnant mare serum gonadotropin.* In the six 29 day old males injected with PMS the average red cell count was 2,760,000 while in the six 32 day old females, similarly treated, the average count was 3,010,000 (see table 3). The four male and six female saline injected controls had average counts of 2,460,000 and 2,660,000 respectively while the normal controls averaged 2,270,000 and 2,480,000. There was very little range

TABLE 3

Effect of PMS gonadotropin on erythrocyte number in juvenile birds

	NO. OF BIRDS	AVERAGE RED CELL COUNT	RANGE	STANDARD DEVIATION
Injected males	6	2.76	2.40–3.36	0.398
Saline injected controls	4	2.46	2.43–3.29	0.125
Normal controls	3	2.27	2.21–2.39	0.083
Injected females	6	3.01	2.71–3.38	0.219
Saline injected controls	6	2.66	2.22–2.98	0.271
Normal controls	8	2.48	2.14–2.75	0.227

Males received 140 I.U. of PMS daily for 24 days and females for 28 days. Injections were begun on the fourth day after hatching.

in the individual birds although the females were consistently slightly higher than the males.

The counts of both saline injected and normal controls were typical of the juvenile level, while those of the treated birds were higher. In the case of the females the average count was well above the normal mature female average, indicating production of androgen by the stimulated juvenile ovary. In addition to a higher blood count, these birds showed evidence of gonad activity in the markedly stimulated comb growth and by crowing, which occurred in males, on the twelfth day of injections. Although this is suggestive evidence a further study of the erythrocyte picture in immature birds stimulated by gonadotropins is desirable.

4. *Estrogen injected capons, poulards and normal males.* The erythrocyte counts of the eleven capons and three bilaterally ovariectomized poulards receiving varying doses of a-estradiol or stilbestrol were, with the exception of capon 3 which had a count of 2,720,000, below the level for a normal female (see

table 4). It was observed in poulard 14, receiving the highest dose of a-estradiol (1.5 mgm.) over a long period of time (43 days), that there was first a fall in the erythrocyte count followed by a rise when the second count was made. In all other cases there was a continued decrease. There appeared no other significant correlation between the dose given and the effect produced. Capons 6 and 8, receiving a-estradiol, showed a greater decrease in red blood cell numbers than capons 9 and 10, receiving the same amounts of stilbestrol for the same length of time. In capon 7 the erythrocyte count returned to the normal capon level when injections were terminated because of sickness of the bird.

The two adult males receiving daily injections of stilbestrol showed a reduc-

TABLE 4

Effect of a-estradiol benzoate and diethylstilbestrol on erythrocyte number

BIRD NO.	SEX*	MGM. DAILY	RED CELLS PER CU. MM.			
			Before	15th day	29th day	43rd day
1	C	0.33 a-estra.	2.36	1.98	2.07**	
2	C	0.33 a-estra.	2.85	2.69	2.22**	
3	C	0.33 a-estra.	2.64	2.72	2.26**	
4	C	0.50 a-estra.			3.65	2.40
5	C	1.00 a-estra.			1.76	1.67
6	C	1.00 a-estra.	3.06	1.90		
7	C	1.50 a-estra.			2.19†	2.57
8	C	1.50 a-estra.	3.40	1.29		
9	C	1.00 stilb.	2.79	2.61		
10	C	1.50 stilb.	2.64	1.51		
11	C	2.00 stilb.	2.74	2.12		
12	BP	0.50 a-estra.			2.30	1.76
13	BP	1.00 a-estra.			1.95	1.37
14	BP	1.50 a-estra.			1.53	1.74
15	R	2.50 stilb.			1.59	1.65
16	R	5.00 stilb.			1.25	1.71

* C., capon; R., rooster; BP., bilaterally ovariectomized poulard.

** Counts made on 25th day of injection, at which time injections were discontinued. Counts 21 days later were 2.53, 3.16, 2.59 respectively, indicating a return to the normal capon level.

† Injections stopped after 21 days because of sickness of bird.

tion in the number of red corpuscles to almost one-third of the normal male number. It is especially interesting to note that these counts are far below the normal female average. The reason for this is not clear although it has been suggested that it may be due to a greater toxicity of stilbestrol.

Here it was also observed that in long continued injections (43 days) the initial drop in erythrocyte number was followed by a subsequent rise. The number, however, remained less than half the normal male count. This secondary rise in the number of erythrocytes, also observed in poulard 14, may be due to an adaptation of the individual to the long continued high dosage resulting in a certain degree of tolerance.

Clotting time was prolonged in all estrogen injected birds. In one of the males receiving stilbestrol, it was noticed that a small wound, made by plucking a feather, continued to bleed for several hours.

These observations suggest that estrogens have an inhibitory effect on the production of erythrocytes, and do not produce their effect merely by neutralizing the stimulus initiated by androgens.

From the results obtained from estrogen injections in dogs (20) and in rats (21), it would seem that the lowered red blood cell number found in estrogen-injected individuals and in females can be accounted for by the action of estrogens on the marrow cavities of the long bones. It has been shown that estrogens cause ossification of the bone marrow, which must seriously interfere with erythrocyte production (3, 4, 22).

If this is the cause of the inhibiting effect of female sex hormones, then we may logically assume that the male hormone causes a stimulation of the erythrocyte-

TABLE 5

Erythrocyte counts in adult intersexual males

TREATMENT	INCUBATION DAY INJECTED	NO. OF BIRDS	AVERAGE RED CELL COUNT	RANGE	STANDARD DEVIATION
0.10 mgm. theelin	4	13	2.96	2.44–4.16	0.454*
0.25 mgm. a-estradiol	5	3	2.92	2.82–3.07	0.108
0.50 mgm. a-estradiol	5	12	2.60	1.87–3.18	0.339
2.50 mgm. stilbestrol	7	3	2.61	2.46–2.75	0.118
2000 I.U. estrogen**	4	5	3.21	2.59–3.63	0.347

* High standard deviation due to one bird with the exceptionally high count of 4.16. (See comment on leukosis in discussion, page 485.) Omitting this bird the average count is 2.86 with a standard deviation of 0.271.

** Amniotin, prepared by E. R. Squibb and Sons from pregnant mare's urine.

forming cells in the marrow of the bones. The nature of the stimulus is not known.

5. *Intersexual males.* The results obtained from counts on adult intersexual males, feminized by estrogen injections during early incubation, are shown in table 5. With the exception of only three counts (4,160,000, 3,630,000, and 3,520,000), all counts were lower than those expected for a normal male. No great differences were observed in the effects of theelin, a-estradiol, a non-crystalline estrogenic substance,[2] and stilbestrol, although the average from birds treated with a-estradiol was slightly lower than the others, and that of birds treated with the non-crystalline estrogenic substance slightly higher. These differences, however, are not statistically significant.

Since the condition of the plumage may be used as a quantitative index of estrogen production (8), these birds were arbitrarily classified into four groups based upon the degree of feminization of their plumage (9). I. Males which

[2] Amniotin, prepared by E. R. Squibb and Sons from pregnant mare's urine.

are essentially masculine in general appearance. II. Males which show a prominent scattering of female feathers on hackle, back, and saddle, and a lesser number of scattered female feathers on the breast. III. Males which have a female or nearly female hackle, back, and saddle, while the breast and tail, though predominantly female, still show many scattered male feathers. IV. Males which are practically indistinguishable from the normal female in general appearance.

The average count of the seven class I birds was 2,837,000, while the average of the six class IV birds was 2,730,000. The two intermediate groups had averages of 2,894,000 (7 birds) and 2,978,000 (10 birds) respectively. The bird with the highest blood count was in plumage group 2. There appeared, therefore, to be no correlation between degree of feminization of plumage and degree of erythrocyte reduction. In the case of the three birds receiving stilbestrol on the seventh day of incubation, the blood counts were similar to those of females, although there was apparently no feminization of plumage. Apparently the quantity of estrogen sufficient to inhibit the production of erythrocytes is below that necessary to produce a change in plumage, indicating a lower threshold for the former.

In the three to five-months-old intersexual male birds, no significant differences were observed in the blood counts of the feminized individuals and the controls, although some of the birds showed distinctly feminized plumage. The average number of erythrocytes in 17 treated males was 2,690,000 and in 4 untreated birds 2,580,000, in both cases typical of the normal juvenile male. Since the number of erythrocytes is normally low until after the sixth month, the effect of the estrogen treatment on erythrocyte production should not be apparent until after that time.

6. *Molting birds.* The coincidence of the low counts in the erythrocytes with juvenile molts reported by Juhn and Domm (13) led us to examine the red cell picture in six sinistral poulards and three intersexual males before and during the molting season. In the poulards the average count before molt was 2,830,000 and during molt 2,710,000. In the intersexual males the pre-molt counts averaged 2,830,000 and the molting counts 2,890,000. In the individual birds no consistent change in blood cell count during the molting period was observed. In some cases there was a rise and in others a fall in the number of erythrocytes, but the average difference between the counts was slight. Although molting appeared to have no effect on the red blood counts, the small number of birds examined does not provide adequate evidence for drawing definite conclusions.

During the course of this work it was noticed that occasionally exceptionally high blood counts would occur in a few individuals in a group of fairly uniform birds. This was seen in three intersexual males, having counts of 4,160,000, 3,163,000 and 3,520,000. The possibility that these birds were suffering from some form of the avian leukosis complex was suggested since previous counts on two paralyzed intersexual males were 3,810,000 and 3,680,000, in both cases higher than the normal male level. However, subsequent observations on five

intersexual males, believed to be leukemic, revealed counts above the average in only two cases.

It is known that the group of pathological conditions known collectively as the avian leukosis complex may manifest itself in various ways, affecting the bones, nerves, viscera, or the blood picture. An increased erythrocyte count has not been reported in connection with the disease, although, in erythroblastosis there is a stimulation in the production of immature cells. However, these do not develop into mature erythrocytes consequently an anemic condition results (12). It seems probable therefore that the increased number of mature erythrocytes found in the four birds examined may represent an early phase of the disease, although our evidence is by no means conclusive.

SUMMARY AND CONCLUSIONS

1. Erythrocyte counts were made on normal males, females, capons, and sinistrally and bilaterally ovariectomized poulards. These averages confirm previously published results.

2. The erythrocyte numbers in capons and bilaterally and sinistrally ovariectomized poulards receiving testosterone were above those of uninjected controls. Twenty-one days after injections were discontinued the erythrocyte numbers had fallen and by forty-five days had reached the normal levels of uninjected capons and poulards.

3. Juvenile males and females, injected with pregnant mare serum, had blood counts higher than their controls.

4. The erythrocyte numbers in normal males, capons, and bilaterally ovariectomized poulards receiving a-estradiol or stilbestrol were below those of normal controls; clotting time was prolonged.

5. Adult intersexual males, feminized by injection of estrogens during incubation, showed lowered blood counts, approximating those of normal hens which could not be correlated with the degree of feminization of plumage.

6. Immature intersexual males, feminized by injection of estrogens during incubation, had the same blood counts as controls, although some of the birds showed feminization of plumage.

7. No significant difference in blood counts before and during molt were observed in the nine fowls examined.

In conclusion, it may be stated that testosterone causes a definite increase in the number of erythrocytes in the blood of the fowl while estrogens cause a decrease, the latter effect probably brought about by changes in bone marrow activity. Our evidence, based on counts in intersexual males, seems to show that the threshold for an estrogen effect on the erythrocyte-forming cells is below that of the plumage.

REFERENCES

(1) Blacher, L. J. Trans. Lab. Exp. Biol., Moscow **1:** 9, 1926.
(2) Blacher, L. J. Biologia Gen. **2:** 435, 1926.
(3) Bloom, M. A., W. Bloom, L. V. Domm and F. C. McLean. Anat. Rec. **78:** 143, 1940.
(4) Bloom, W. and L. V. Domm. Anat. Rec. **81:** 91, 1941.

(5) CASTRODALE, D., O. BIERBAUM, E. B. HEILWIG AND C. MACBRYDE. Endocrinology **29:** 363, 1941.
(6) CHAUDHURI, A. C. Proc. Roy. Physiol. Soc. **21:** 109, 1926.
(7) CRAFTS, R. C. Endocrinology **29:** 606, 1941.
(8) DOMM, L. V. Sex and internal secretions. 2nd ed., Chapter V, 227, 1939.
(9) DOMM, L. V. AND D. E. DAVIS. Proc. Soc. Exper. Biol. and Med. **48:** 665, 1941.
(10) DOMM, L. V. AND W. BLOOM. Anat. Rec. **81:** 91, 1941.
(11) FORKNER, C. E. J. Exper. Med. **50:** 121, 1929.
(12) HALL, W. S. U. S. Dept. Agric. Circular 628, 1942.
(13) JUHN, M. AND L. V. DOMM. This Journal **94:** 656, 1930.
(14) LANDAUER, W. AND L. T. DAVID. Folia Haemotologia **50:** 1, 1933.
(15) MCCULLAGH, E. P. AND R. JONES. Cleveland Clin. Quart. **8:** 79, 1941.
(16) NICE, L. B., M. M. NICE AND R. M. KRAFT. Wilson Bull. **47:** 120, 1935.
(17) RIDDLE, O. AND P. E. BRAUCHER. This Journal **108:** 554, 1934.
(18) TABER, E., D. E. DAVIS AND L. V. DOMM. Anat. Rec. **81:** 89, 1941.
(19) TYSLOWITZ, R. AND C. G. HARTMAN. Endocrinology **29:** 349, 1941.
(20) TYSLOWITZ, R. AND E. DINGEMANSE. Endocrinology **29:** 817, 1941.
(21) VOLLMER, E. P. AND A. S. GORDON. Endocrinology **29:** 828, 1941.
(22) ZONDEK, B. Folia Clin. Orient. **1:** 1, 1937.

THIAMINE AND THE SPECIFIC DYNAMIC ACTION OF CARBOHYDRATE AND FAT

GORDON C. RING

From the Department of Physiology, The Ohio State University, Columbus

Received for publication August 20, 1942

It has been suggested that the specific dynamic action of carbohydrate is due to the heat expended in its intermediary metabolism (see Dann and Chambers, 1930). This intermediary metabolism may result in the formation of glycogen or the production of fat. One would expect that the synthesis of fat from sugar would require more energy than the production of glycogen. Now, since thiamine stimulates new formation of fat (see Whipple, Church and Stevens, 1937; McHenry and Gavin, 1938, 1939), the S.D.A. of glucose should be greater when thiamine is given. In the present study, a comparison has been made between the S.D.A. of glucose given alone, or glucose and thiamine, and of glucose, thiamine and fat. The last mixture was used because it was thought that fat given with glucose plus thiamine would depress the new formation of fat, and therefore, the S.D.A. However, the S.D.A. of fat itself complicates the interpretation of results.

METHOD. The procedure followed was similar to that described in an earlier paper (Ring, 1942). The rats were given the usual diet of Purina Dog Chow. Records of post-absorptive metabolism were obtained for a period of three hours. The rats were then removed from the apparatus and one of the following solutions or mixtures was given by stomach tube:

Three cubic centimeters 50 per cent glucose
Three cubic centimeters 50 per cent glucose plus 50 gamma of thiamine
Three cubic centimeters 50 per cent glucose, 50 gamma of thiamine, 1½ cc. of oleic acid
Three cubic centimeters water plus 50 gamma of thiamine
Three cubic centimeters water, 50 gamma of thiamine, 1½ cc. oleic acid.

After returning the rats to the metabolism apparatus, the oxygen consumption was continuously measured during the succeeding seven hours. Quiet periods were selected each hour and averaged to determine the resting oxygen consumption during this period. The first three solutions were given in rotation at weekly intervals until nine observations had been made. A group of eight rats was kept on this regime. Using a second group, the metabolic effect of the last two mixtures was studied. To estimate the heat production, the respiratory quotients were determined by using the Haldane principle in one quarter of the experiments. Calculations of heat production were based on these figures.

RESULTS. In reporting our findings, the result for each separate experiment is not presented. Only the average increase in metabolism for the three days when the same material was given is to be found in table 1. Thus the grand

average at the bottom of each column and the probable error were calculated from a total of 24 observations. It is clear that the S.D.A. of glucose varies widely in different rats. However, in every rat studied the thiamine increased the metabolic effect of the glucose. This is not due to any marked effect of the thiamine alone on metabolism. The metabolic effect of giving 50 gamma of thiamine dissolved in 3 cc. of water has not been worked out with great care but 22 observations made on 11 rats showed an average increase of 0.9 per cent. Since the difference between the S.D.A. of glucose and of glucose plus thiamine is 3.8 per cent, it seems highly probable that this effect of the thiamine is specifically on the glucose. At any rate, there is less than one chance in 10,000 that the difference of 3.8 per cent is not significant. If this increased S.D.A. is due to the conversion of sugar into fat, then the respiratory quotients should be

TABLE 1

Average percentage increase in metabolism during seven hours after ingestion of food

(Each figure is the average of 3 observations)

RAT NO.	GLUCOSE*	GLUCOSE AND THIAMINE**	GLUCOSE, THIAMINE† AND OLEIC ACID
1	4.8	5.2	2.7
2	8.2	9.4	14.7
3	3.3	9.9	6.4
4	4.5	9.6	5.9
5	1.9	9.0	12.3
6	2.5	10.6	7.0
7	4.9	5.6	2.7
8	3.8	4.6	2.1
Average.....	4.2 ± 0.4	8.0 ± 0.5	6.7 ± 1.0

* Three cubic centimeters 50 per cent glucose.

** Three cubic centimeters 50 per cent glucose plus 50 gamma of thiamine.

† Three cubic centimeter 50 per cent glucose, 50 gamma of thiamine, 1½ cc. oleic acid.

raised by the addition of thiamine to glucose. This was found to be the case. The respiratory quotients during the seven hours after giving glucose without thiamine averaged 0.803 for this group of rats. (It was 0.796 for similar experiments a year ago.) When thiamine was added, the quotient rose to 0.812. When the metabolism was measured for a shorter period of time, the results suggested that thiamine produced its greatest rise in respiratory quotients during the latter half of the seven hour period.

In six of the eight rats studied, the addition of oleic acid to the mixture of glucose and thiamine reduced the S.D.A. Although this reduction is possibly not significant, it is probable from statistical analysis that the addition of fatty acid does not increase the S.D.A. above that of glucose plus thiamine. Yet one would hardly think that the S.D.A. of the fat would be completely absent. Previously, Ring (1942) showed that the S.D.A. of fat plus glucose is greater than that of glucose alone. We, therefore, believe that the addition of fat to the

mixture of glucose and thiamine has depressed the effect of thiamine in stimulating the conversion of sugar into fat. The lower respiratory quotient of 0.797 found after fat was added supports this impression, though other explanations are possible.

It is unlikely that thiamine has any effect on the metabolism of oleic acid. Measurements of the S.D.A. of oleic acid plus thiamine averaged, in eight rats, 6.9 per cent. Previous averages for other rats given oleic acid alone were between 6.6 and 8.7 per cent.

The results obtained support our previous contention (1942) that the S.D.A.s of the various foodstuffs are not additive when two or more are given at the same time. The S.D.A. of fat, glucose and thiamine proved to be 6.7 per cent, or less than half the 14.9 per cent which totaling the individual responses gives. These findings substantiate the theory that the S.D.A. of carbohydrate is due to the energy wasted in intermediary metabolism. That energy is required to convert the foods into a form suitable for storage.

The differences obtained in these experiments are not large and it might be argued that more striking results could have been obtained if the animals studied had been deficient in thiamine. This may be true but such deficient animals have a lowered metabolism (see Göthlin, 1938). Since the thiamine alone would restore the metabolism to normal, it is quite probable that this complication would offset the benefit of studying an increased conversion of carbohydrate into fat.

CONCLUSIONS

1. The specific dynamic effect of glucose (4.2 per cent) is less than that of glucose and thiamine (8.0 per cent) (see table 1).

2. Thiamine in water has very little if any effect upon the basal metabolism (0.9 per cent). When thiamine is added to glucose, the extra energy observed is probably expended in converting the sugar into fat. The fact that the respiratory quotient is higher after giving thiamine and glucose than after glucose alone supports this suggestion.

3. When oleic acid is added to glucose and thiamine, the specific dynamic effect (6.7 per cent) is not increased over that of the glucose and thiamine (8.0 per cent). Unless the oleic acid has no specific dynamic action, the effect of thiamine in stimulating conversion of carbohydrate into fat must be reduced as the lowered respiratory quotient suggests.

4. The specific dynamic effect of oleic acid appears not to be affected by giving thiamine.

REFERENCES

(1) Dann, M. and W. H. Chambers. J. Biol. Chem. **89:** 675, 1930.
(2) Whipple, D. V., C. F. Church and H. Stevens. Am. J. Med. Sci. **193:** 733, 1937.
(3) McHenry, E. W. and G. Gavin. J. Biol. Chem. **125:** 653, 1938; **128:** 45, 1939.
(4) Ring, G. C. This Journal **135:** 742, 1942.
(5) Göthlin, G. F. Skand. Arch. Physiol. **80:** 133, 1938.

REACTIONS OF THE AORTA IN HEMORRHAGIC HYPOTENSION AND SHOCK[1]

CARL J. WIGGERS, RENE WEGRIA AND NEIL D. NICKERSON

From the Department of Physiology, Western Reserve University Medical School, Cleveland, O.

Received for publication October 1, 1942

It is fairly common knowledge among experimentalists that a laboratory procedure designed to induce shock may, for hours, cause only a moderate circulatory imbalance; then quite rapidly blood pressure declines, the heart starts to slow, the pulse pressure decreases and a state or circulatory failure develops which is no longer benefited permanently by transfusions. Such a turning point is generally discernible in hemorrhagic shock which develops after a protracted period of hypotension and subsequent reinfusion of the blood withdrawn (1).

The factors or processes responsible for the phenomenon of irreversibility remain an enigma. Many can be suggested, but none has been incriminated as the only or dominant one.

Since such a circulatory turning point is generally characterized by a marked reduction in pulse pressure, a fairly sudden decrease in cardiac output may be concerned. However, the ratio, systolic discharge/aortic capacity, can be reduced equally by decreasing the numerator or by increasing the denominator. Consequently, it is also conceivable that a fairly sudden enlargement of the aorta due to failure of its intrinsic musculature might account for such pulse pressure changes. Since a number of investigators (for review cf. Bazett (2)) have recently stressed the importance of active changes in aortic capacity and distensibility, and since we (3) unexpectedly found that the aorta decreases in size and increases in extensibility during active hypertension, one of us (4) suggested that "the remote possibility that failure of mechanisms which adapt the size and elasticity of the aorta to changing volumes and pressures of blood may be a decisive factor in circulatory failure needs to be investigated." This communication seeks to report, very briefly, results from three types of experiments which lend *no support* to such a suggestion.

Aortagraph experiments. Eight experiments were performed on anesthetized dogs under mild artificial respiration in the following manner. With the animal on its right side, the left side of the chest was opened by an intercostal incision and forcible retraction of ribs, sufficient to make the upper portion of the ascending aorta accessible. An aortagraph similar to that previously described (3) was stitched to the aorta in line with its diameter and connected by a leakproof tubing with a Frank segment capsule. The optical record magnified changes approximately 10 to 12 times. An idea of the sensitivity is gained from calculation ($\pi r^2 h$) that in an aorta 30 cm. in length and 1.3 cm. in diameter a change in diameter of 1 mm. (or 1 cm. of record) causes, at normal

[1] Supported by a grant from the Commonwealth Fund.

pressures, a volume change of about 5 cc. or 15 per cent of its total volume. Intraaortic pressures were recorded simultaneously with a calibrated optical manometer of the Gregg type.

In general, our experimental plan consisted in reducing arterial pressures to about 50 mm. Hg by fairly rapid bleeding and after maintenance of such pressure for an hour or more to reduce it approximately to 30 mm. for another 45 minutes. Thereupon, all the withdrawn blood was reinfused and the state of progressive circulatory failure awaited.

Our results indicated (1) that during an acute hemorrhage by which mean pressure was reduced from 130–140 to 50–40 mm. Hg, the diastolic and systolic diameters of the aorta diminished directionally as intra-arterial pressures. The form of the pulse also changed with that of the intra-aortic pressure pulse. Since the natural volume elasticity of the aorta was unknown, it was impossible to determine whether such reduction involved more than an elastic retraction. Without this knowledge, continued observations of qualitative directional changes might still have proved useful had it not been for unanticipated difficulties in preventing displacement of such an aortagraph. Our chief difficulty consisted in its dislocation by the assumption of vigorous spontaneous breathing during the low blood pressure period. Obviously, one such displacement during the day's experiment sufficed to alter recorded relationships of aortic size. Consequently, it was necessary to restrict comparisons of aortic diameter/diastolic pressure to certain portions of our experimental day.

During the period of marked hypotension, when processes which later manifest themselves as shock are presumably operating, discordant directional changes in aortic or diastolic pressure never occurred. On reinfusion of the withdrawn blood the diastolic size increased *pari passu* with diastolic pressure and the amplitude of the aortagraph pulses increased with the intra-aortic pulse pressure. In three experiments, however, the aortic diameter decreased a few minutes after blood infusion had stopped, while intra-aortic pressure remained unaltered. This resembled the changes induced by acute hypertension, but was much less in magnitude. It is possible that such effects are mediated without intervention of nerve elements and correspond to the stretch stimulus reactions reported by others (5) in the case of "muscular arteries." During the progressive decline of blood pressure following transfusion, most of our experiments revealed no changes that could not be assigned to passive retraction. In two, however, oscillatory changes of diastolic size and pulse amplitude occurred which were not related to intra-aortic pressure changes.

While such occasional discordances between aortagrams and pressure pulses are perhaps suggestive of occasional active changes in the aorta, their absence in most experiments, their variability and lack of relation to critical periods of circulatory failure prevent us from attaching significance to them as factors influencing the downward course.

Since such studies of the aorta are entirely qualitative and since even a single adjustment during a day's experiment left us without information as to how diastolic diameters corresponded during the initial period of low pressure and after development of shock we turned to another method of study.

Circumferometer experiments. This procedure consisted in measuring directly, by a specially constructed instrument called a circumferometer, changes in the circumference of a definite region of the descending aorta during different phases of an experiment on hemorrhagic hypotension and shock. In addition to making comparisons of aortic circumference/aortic diastolic pressure at all times, the procedure had the advantage of a measure of comparative quantitation. Thus, by vagal slowing and stoppage of the heart—which lowered pressures from say 100 to 20 mm. Hg—it was possible to construct a control curve of circumference/pressure relations, which could perhaps be assumed to represent passive retraction. However, since Dow and Hamilton (6) presented circumstantial evidence that vagus stimulation may affect elasticity of the aorta by action on its muscular elements, the possibility that it also affects the circumference/pressure relations must be kept in mind. At any event, circumfer-

Fig. 1

ence/pressure relations occurring during different phases of the experiment could be compared with a definite curve.

The circumferometer, shown in figure 1, had a fixed and a sliding arm to which an inelastic thread looped around the aorta was attached. The sliding arm operated as a rack geared to a pinion, and to this a pointer was attached. For each measurement, the sliding arm was drawn up until the loop fitted snugly around the aorta during diastole and gave a slight systolic shock to the controlling finger, and the circumference thus determined was read on a dial. Since changes in circumference were approximately 3× as great as changes in diameter and as the scale magnified four times, a total magnification of 12 was realized. However, the readings necessarily depended on personal tactile and visual judgments as to when a "just snug" fit of the encircling thread was achieved. In each instance, a number of measurements were made by each of several experimenters and agreement was usually reached as to the exact measurement.

Measurements of aortic diameter were made frequently throughout six experiments on hemorrhagic shock. In five of these the circumference/pressure

relations during, and immediately after, hemorrhage corresponded rather well with the standard curve derived during vagal inhibition of the heart. In one experiment only, the circumference decreased more. However, neither in this nor in other experiments did we find at any later stage of hemorrhagic hypotension, reinfusion, or final circulatory failure any deviations which were significant enough to indicate that active changes in the aorta participated in process of shock production. Certainly, no sudden or progressive changes were detectable during late periods of severe hypotension when the processes producing irreversibility probably operate to a marked degree. If our method was too crude to detect smaller changes they could not have played a prominent role in producing the characteristic hemodynamic alterations at the crisis. Admittedly, the remote possibility existed that active changes in the aorta were prevented by influence of the previous operative procedures, or that such changes had already taken place before our measurements started.

Pulse conduction rates. To test the foregoing possibility, we studied, in animals submitted to minor operative procedures, only, the changes in the subclavian-femoral conduction rates at equivalent diastolic pressures at various periods of shock experiments. For this purpose we surveyed a large number of records from experiments already reported (1, 7, 8) in which subclavian and femoral pulses had been recorded and in which equivalent diastolic pressures were fortuitously established for brief intervals at different stages of the experiment. In twelve such experiments, measurements failed to reveal significant differences in such conduction times at diverse stages of the experiments; indeed, in half of the records, pulse conduction times were identical.

SUMMARY AND CONCLUSIONS

On the basis of negative evidence from three modes of experimental approach, we are forced to conclude that it appears highly improbable that active changes in the aortic wall play any rôle in the initiation or progression of hemorrhagic shock or in the establishment of an irreversible state.

REFERENCES

(1) Werle, J. M., R. S. Cosby and C. J. Wiggers. This Journal **136:** 401, 1942.
(2) Bazett, H. C. Ann. Rev. Physiol. **1:** 163, 1939.
(3) Wiggers, C. J. and R. Wegria. This Journal **124:** 603, 1938.
(4) Wiggers, C. J. Physiol. Rev. **22:** 74, 1942.
(5) Bayliss, W. M. J. Physiol. **28:** 220, 1902; Wachholder, K. Pflüger's Arch. **190:** 222, 1921.
(6) Dow, P. and W. F. Hamilton. This Journal **125:** 60, 1939.
(7) Wiggers, C. J. and J. M. Werle. This Journal **136:** 421, 1942.
(8) Wegria, R., A. Guevera Rojas and C. J. Wiggers. This Journal **138:** 212, 1943.

CAPILLARY PERMEABILITY TO INTRAVENOUSLY ADMINISTERED GELATINE

J. MAXWELL LITTLE AND HERBERT S. WELLS

From the Department of Physiology and Pharmacology, Bowman Gray School of Medicine, Winston-Salem, N. C.

Received for publication October 7, 1942

With the renewed interest in gelatine-saline as a possible blood substitute (1, 2), the question of its rate of disappearance from the blood becomes important. In his review of the literature Amberson (3) states that gelatine undoubtedly leaves the blood stream with fair ease. This statement is based upon indirect estimations, made by several investigators, of the concentration of gelatine in blood at varying times following its injection into normal animals.

There are no data on the rate of passage of gelatine through the capillary wall, but one might suppose that it passes into the tissue fluids rather readily in view of its reported rapid disappearance from the blood. We have tested this possibility by determining the relation between the concentrations of gelatine and serum proteins in the blood and in a capillary filtrate obtained by irritation of the intestine by manipulation.

METHOD. Dogs were anesthetized with pentobarbital sodium. The carotid artery was cannulated for recording the mean blood pressure and for bleeding and injections. A mid-line abdominal incision was made through which the small intestine could be withdrawn for manipulation and the collection of fluid.

The animals were bled rapidly a quantity approximating 3 to 4 per cent of their body weight and were immediately transfused with a similar volume of normal saline. The animals were bled again to the same extent and were transfused with cells suspended in a minimum volume of normal saline plus a gelatine-saline solution sufficient to replace the volume of blood withdrawn. The cells were obtained from a donor dog and were washed twice with saline. The gelatine prepared[1] by hydrolysis of alkali-treated bone collagen was given as a 6 or 8 per cent solution in normal saline. In experiment 5 this solution was adjusted to pH 7.3. In experiments 4 and 5 the solution was autoclaved at 15 pounds' pressure for twenty minutes.

Loops of small bowel of varying lengths were removed from the peritoneal cavity, and the serous surface was irritated by pinching, resulting in the flow from the serous surface of fluid presumably derived from injured capillaries. As shown by Beard and Blalock (4) vigorous mechanical manipulation (pinching) results in the production of a fluid having a serum protein concentration equal to that of the blood. This type of irritation was used in all experiments except the first two, in which a milder degree of irritation was produced by

[1] Supplied by Knox Gelatine Co., Johnstown, N. Y. and Camden, N. J. Lot number B 78-1's.

very light pinching following the application of sodium chloride crystals to the gut surface.

Following irritation, the loops of bowel were coiled in a beaker arranged to avoid traction on the mesentery. The beaker and contents were kept warm by an electric lamp. Evaporation was minimized by covering the beaker with a sheet of rubber dam. The first 5 ml. of fluid collected in the beaker were discarded, the surface of the gut was wiped with dry sponges, then 8 to 10 ml. samples of fluid were collected for analyses. Blood samples were taken at the beginning and end of a fluid collection period in most cases, but in some the blood samples were taken before and after two periods of fluid collection or at the middle of a period.

For the determination of gelatine 2 ml. of serum or fluid were diluted with 7 ml. of water and 1 ml. of 50 per cent trichloracetic acid (1) was added. One ml. aliquots of the filtrate were taken for nitrogen determinations. The non-protein nitrogen was determined on 10 ml. aliquots of the supernatant liquid obtained by centrifuging the tungstic acid precipitate from a solution consisting of 4 ml. of serum or fluid, 2 ml. of 10 per cent sodium tungstate, 2 ml. of $\frac{2}{3}$ N sulphuric acid and 32 ml. of water. The total nitrogen was determined on 1 ml. aliquots of a 1:10 dilution of serum or fluid. The procedures of Van Slyke (5) and Van Slyke and Kugel (6) were used for all nitrogen determinations. The usual factor of 6.25 was used to convert nitrogen values into serum protein or gelatine values. All determinations were done in duplicate, and when the variation between duplicates exceeded 3 per cent the determination was repeated.

Results and discussion. The results are summarized in table 1. The figures in the last column indicate the ease with which plasma gelatine passes through the capillary walls relative to the ease with which serum proteins pass through. If both pass through with equal facility the value of $\frac{R_2}{R_1}$ will be unity, but if gelatine passes through less readily the ratio will be less than unity.

It will be seen that the figures in the last column are all less than unity with an average value of 0.58 and with a spread of 0.47–0.64. We interpret this to mean that either all gelatine passes through injured capillary walls at about 0.6 the rate at which serum proteins pass through, or that approximately 35 to 60 per cent of the gelatine consists of particles completely unable to pass through capillaries with the degree of injury encountered in these experiments (see R_2 in table 1). If the first interpretation is correct it is true both when capillary damage is severe enough to allow all plasma proteins to pass through ($R_1 = 1$) and when less severe damage results in only a fractional loss of plasma proteins ($R_1 < 1$), as will be seen by comparing the values for R_1 and R_2/R_1.

We have considered the possibility that the fluid which we obtained is not representative of the capillary filtrate, for during its passage from the capillaries through tissue spaces to the serous surface of the gut the original filtrate might be altered in several ways. First, it might be diluted with preformed tissue fluid containing a relatively high concentration of plasma proteins. Secondly, some of the gelatine might be completely hydrolyzed. Thirdly, a part of the gelatine might be utilized by the local tissues.

In experiment 6, the first fluid sample was collected 2.6 hours after gelatine was injected. The intestinal loops were returned to the peritoneal cavity and vigorously pinched at intervals during the next 3 hours to remove any preformed

TABLE 1

EXPERIMENT NO.	SAMPLES	TIME	SERUM PROTEIN	GELATINE	NON-PROTEIN NITROGEN	SAMPLES USED FOR CALCULATING RATIOS (f: FLUID; s: SERUM)	RATIO OF FLUID PROTEIN TO SERUM PROTEIN CONCENTRATION	RATIO OF FLUID GELATINE TO SERUM GELATINE CONCENTRATION	$\frac{R_2}{R_1}$
		*minutes**	*gram per cent*	*gram per cent*	*mgm. per cent*		R_1	R_2	
1	Serum 1	30	3.43	1.57	27.1				
	Fluid 1	30–46	2.36	0.63	27.2	f_1, s_1	0.69	0.40	0.58
	Serum 2	55	3.41	1.54	28.5				
	Fluid 2	46–55	2.70	0.71	27.2	f_2, s_2	0.79	0.46	0.58
2	Serum 1	35	2.52	1.97	30.1				
	Fluid 1	35–75	2.01	0.86	33.7	f_1, s_1, s_2†	0.85	0.47	0.55
	Serum 2	70	2.21	1.68	35.0				
3	Serum 1	40	1.55	3.09	28.8				
	Fluid 1	40–65	1.58	1.85	27.6	f_1, s_1	1.02	0.60	0.59
	Fluid 2	65–95	1.14	1.49	28.1	f_2, s_2	0.85	0.54	0.64
	Serum 2	95	1.35	2.74	28.8				
	Fluid 3	95–160	1.54	1.33	29.8	f_3, s_2	0.99	0.50	0.51
	Serum 3	160	1.56	2.64	29.7				
4	Serum 1	44	1.71	2.62	27.2				
	Fluid 1	44–90	1.77	1.65	29.5	f_1, s_1, s_2†	1.02	0.65	0.64
	Serum 2	90	1.76	2.49	29.3				
	Fluid 2	92–124	1.89	1.57	31.9	f_2, s_2, s_2†	1.04	0.64	0.62
	Serum 3	124	1.88	2.43	30.1				
5	Serum 1	55	0.93	2.79	35.8				
	Fluid 1	55–65	1.06	1.50	33.5	f_1, s_1	1.14	0.54	0.47
	Fluid 2	65–77	1.09	1.57	33.9	f_2, s_2	1.07	0.62	0.58
	Serum 2	77	1.02	2.53	31.7				
	Fluid 3	104–128	1.00	1.52	36.7	f_3, s_2	0.98	0.60	0.61
	Serum 3	128		2.51	34.8				
6	Serum 1	145	2.15	2.05	26.5				
	Fluid 1	125–180	2.10	1.12	29.1	f_1, s_1	0.98	0.55	0.56
	Fluid 2	372–425	2.76‡	1.19	48.0	f_2, s_2	1.12	0.64	0.57
	Serum 2	402	2.46	1.87	48.6				

* Time represents minutes after injection of gelatine.
† The average of two serum values used.
‡ Considerable hemolysis in sample.

tissue fluid. A second fluid sample was collected 4 hours after the first. If dilution with preformed tissue fluid is the factor responsible for our results, the second fluid sample should have a value for R_2 at least approaching unity. It will be seen that this is not the case.

If the gelatine undergoes hydrolysis after the fluid leaves the vascular system, one would expect to find elevated non-protein nitrogen values for the fluid. This is not the case.

It is very unlikely, although difficult to prove, that gelatine would be utilized at a faster rate by the tissues through which the fluid passes than would be the serum proteins which are also present. It would be even more unlikely that the rate of utilization would be so constant from one animal to another.

Therefore the data indicate that none of these possibilities are likely, and that we have, in fact, analyzed a fluid which is probably very nearly identical with the true capillary filtrate.

One must also consider the possibility that the fraction of the plasma gelatine which leaves injured capillaries will also pass through normal capillaries. If this were true one would expect that in those experiments (1 and 2) in which the capillary injury is not sufficient to permit complete passage of serum proteins R_2 would still be equal to R_2 in those experiments (3, 4, 5 and 6) in which serum proteins passed through completely. This is not the case, for in the former experiments the average of R_2 is 0.44 while in the latter the average of R_2 is 0.59.

It is of interest to note that autoclaved gelatine (expts. 4 and 5) does not pass through the walls of the injured capillaries more readily than unautoclaved gelatine (expts. 1, 2, 3 and 6). This is surprising in view of unpublished evidence obtained in this laboratory that autoclaving results in some hydrolysis of the gelatine, as indicated by a 40 per cent increase of its colloid osmotic pressure. One would expect, if the retention of gelatine by capillaries is due to the large size of the particles, that hydrolysis would allow more to escape. Since no more does escape one can perhaps assume either that the hydrolysis involves chiefly particles originally small enough to pass through readily, or that very large particles are split into units still too large to escape.

SUMMARY

It has been shown that intestinal capillaries injured sufficiently to permit the partial or complete passage of serum proteins through their walls allow the passage of only 35 to 60 per cent of plasma gelatine. This is thought to be due either to a slower rate of escape for gelatine than for serum proteins or to the presence of gelatine particles to which the injured capillary is completely impermeable.

We wish to acknowledge with appreciation aid given by Mr. J. E. Atkins, Jr., who assisted in some of the preliminary experimental work.

REFERENCES

(1) WATERS, E. T. Canad. M. A. J. **45:** 395, 1941.
(2) GORDON, H., L. J. HOGE AND H. LAWSON. Am. J. M. Sc. **204:** 4, 1942.
(3) AMBERSON, W. R. Biol. Rev. **12:** 48, 1937.
(4) BEARD, J. W. AND A. BLALOCK. Arch. Surg. **22:** 617, 1931.
(5) VAN SLYKE, D. D. J. Biol. Chem. **71:** 235, 1927.
(6) VAN SLYKE, D. D. AND V. H. KUGEL. J. Biol. Chem. **102:** 489, 1933.

STUDIES IN EXPERIMENTAL TRAUMATIC SHOCK WITH PARTICULAR REFERENCE TO PLASMA POTASSIUM CHANGES[1]

JEANNE F. MANERY AND D. Y. SOLANDT

From the Departments of Biochemistry and Physiology, University of Toronto, Toronto, Canada

Received for publication August 12, 1942

The research reported here was undertaken in order to investigate the suggestion made by Scudder (12) that potassium might be a toxic factor in shock. A critical examination of much of the data on which this suggestion was based, particularly that dealing with traumatic shock (2, 14), seemed to us to be inconclusive because of the small number of animals used and the variability of the increase in plasma potassium reported. Since one hundred grams of muscle, kidney, liver or brain contain about 400 mgm. of potassium, there is reason to suspect mobilization of a considerable amount of the base from damaged tissue. Winkler, Hoff and Smith (13) showed that 500 mgm. of potassium killed a 10 kgm. dog when injected intravenously over a period of 13 minutes. Furthermore shock has been produced frequently by administration of saline or aqueous extracts of tissues, by tissue autolysates and implants, each of which might contain appreciable amounts of potassium. Recently Pen, Campbell and Manery (11) demonstrated that potassium was the toxic factor in certain alcoholic extracts of muscle which were found to cause death in mice.

We have analysed the plasma in a large series of dogs in which traumatic shock with some hemorrhage was produced by the method employed by Best and Solandt (1). In addition to potassium analyses, measurements of local fluid loss and red cell volume, the water content and chloride concentration of plasma were also estimated. Some sodium analyses were performed, thereby allowing a comparison with the condition of adrenal insufficiency.

In brief, the results demonstrated that the potassium concentration of the plasma increased in a variable manner following muscular trauma. In most cases some decrease in chloride concentration was observed. Although the electrolyte changes in this type of shock resemble those in adrenal insufficiency qualitatively, they differ so greatly quantitatively that there is little reason at present to assume a similarity of causes.

METHOD. The condition of shock was produced in dogs (5–9 kgm.), under light ether anesthesia, by inflicting 1000 to 1500 light blows with a rubber mallet on a small fleshy area near the posterior margin of each thigh. This required $\frac{1}{2}$ to $\frac{3}{4}$ of an hour and the tissue in the region of injury usually felt soft and spongy at its termination. Dissection at death revealed that the muscle was not pulped as had occurred in the earlier experiments of Best and Solandt but that the

[1] The data reported here were presented at the meeting of the American Physiological Society, Chicago, 1941 (This Journal **113**: P376, 1941).

spongy texture was due to fluid accumulation in the subcutaneous and intermuscular fat and connective tissue. On rare occasions the fibers of a muscle bundle directly below the pounded area were found to be severed transversely, but elsewhere in the same region the muscles were intact although usually red and swollen in appearance. The animals recovered from anesthesia and were bright and active for about 3 to 17 hours (average = 8.7 hr.) when they succumbed in secondary shock. The condition was manifested by weakness, a fall in blood pressure and final prostration; the blood pressure was measured as rapidly as possible before death by connecting a cannula in the carotid artery to a mercury manometer.

Blood samples were taken from the external jugular vein before anesthesia and at varying intervals following the muscle trauma. Jugular vein plasma was chosen to represent blood coming *from* a tissue and hence least likely to exhibit potassium changes since the tissue might be expected to absorb some of the incoming potassium. As the blood pressure fell in shock it was necessary to procure the terminal sample from the carotid artery and the heart. The heparinized blood was centrifuged at once, the hematocrit values noted and the plasma removed from the cells. Chloride was determined on 0.3 to 0.5 ml. of plasma. Samples of 0.5 to 1.0 ml. of plasma were weighed in platinum crucibles and dried in an oven overnight at 100°C in order to estimate the water content. A little sulfuric acid was then added to the crucibles and the plasma samples ashed in a muffle furnace overnight at about 550°C. It was found that the Shohl and Bennett potassium method as described by Fenn et al. (6), when modified slightly, could be used satisfactorily on such small samples. The average difference between 39 duplicate samples was 2.8 per cent. Chloride was determined by the Van Slyke procedure as used by Manery, Danielson and Hastings (8) and sodium by the Butler and Tuthill technique (4).

Results. 1. *Potassium.* (a) *Plasma K in control dogs.* The concentration of potassium in dog plasma drawn in the mornings (table 1) from the external jugular vein ranged from 12.2 to 20.0 mgm. per 100 ml. in 44 normal dogs with an average value of 15.9 mgm. (p.e. ± 1.7 mgm.). This range of individual variation from one dog to another is somewhat less than others previously reported (2, 9). To estimate the extent of the variation in any one animal, two pre-anesthetic samples were taken 20 to 35 minutes apart, from each of 14 dogs. The average difference between the two samples was 5.4 per cent (p.e. = ± 3.2 per cent). In 8 cases the second sample showed a lower potassium value than the first (see dogs 18, 24 and 26, fig. 2), which may be due to the adrenalin secreted as a result of the first venous puncture. A single injection of adrenalin has been shown to cause an immediate and transitory rise in plasma potassium followed by a secondary and somewhat more prolonged fall (7). In the cases in which blood was drawn from the same animal on several successive days the plasma potassium showed remarkable constancy from day to day.

Control experiments were performed by exposing dogs to ether anesthesia for ½ to 1 hour, allowing them to recover and withdrawing blood at intervals throughout the day. The complete data from 4 representative cases are

TABLE 1

Plasma potassium concentrations and changes in hematocrit values (ΔC.V.) (in per cent of the initial value) following muscular trauma

DOG NO.	DOG WT.	SAMPLE 1		SAMPLE 2			SAMPLE 3			SAMPLE 4			SURVIVAL TIME
		K	C.V.	Time	K	ΔC.V.	Time	K	ΔC.V.	Time	K	ΔC.V.	
					Group A—Died. Terminal sample obtained								
	kgm.	*mgm. per cent*	*per cent*	*hr.; m.*	*mgm. per cent*	*per cent*	*hr.; m.*	*mgm. per cent*	*per cent*	*hr.; m.*	*mgm. per cent*	*per cent*	*hr.; m.*
5	5.7	19.6	51				*2; 30	44.5	−20	†2; 40	47.3	−20	2; 40
6	8.6	15.9	52	3; 45	15.8	+10	*5; 25	33.4	+10	†5; 35	42.0	+13	5; 35
7	6.4	17.0		1; 5	14.8		5; 0	17.0		†6; 25	44.0		5; 50
8	7.7			4; 15	14.7					6; 15	26.9		6; 17
4	9.1	14.1		4; 25	17.3		5; 25	17.5		6; 31	25.7		6; 36
9	7.3	16.8	60	5; 20	17.3	−25	*7; 10	21.1	−19	†7; 30	30.6	−18	7; 30
10	7.7			1; 39	16.1		5; 40	18.5		7; 25	19.6		8; 35
11	5.7	15.3	38	5; 20	15.2	−16	8; 10	25.0	−11	†8; 35	33.8	−19	8; 35
12	6.1	17.5	58	6; 35	19.0	−10	*10; 5	21.9	−11	†10; 14	23.0	−11	10; 14
13	5.0	16.9	34	6; 5	22.8	−28				16; 50	20.5		17; 5
14	4.5	14.6	50	7; 45	24.6	−5	*17; 0	23.3	−2	†17; 35	46.4	+2	17; 35
					Group B—Died. No terminal sample obtained								
15	12.7	18.4	47	1; 35	13.0	−11	3; 50	15.8	−11				5; 35
16	7.0	15.6	48				4; 30	15.3	−8				5; 45
17	7.7	18.5	48	1; 55	20.6	+4							7–9;
18	8.6	16.3		2; 40	16.9		4; 48	19.9					8; 40
19	5.2	17.0	50	2; 40	21.3	−7							10; 50
20	5.4			7; 15	22.1								8–10;
21	7.3	15.5		0; 45	20.4		7; 35	27.4					8–16;
					Group C—Lived								
22	7.5	14.5		1; 15	16.6		5; 15	14.5		8; 10	14.3		Lived
23	6.8	16.1	39	1; 35	15.6	+4	5; 20	15.9	−9				Lived
24	8.2	16.4		3; 0	19.1		7; 40	19.6		10; 35	18.3		Lived
25	6.8	12.8		1; 7	15.0		8; 5	16.6					Lived
26	7.3	16.6	58	4; 50	17.3	+3	10; 20	17.6	+5	14; 0	20.2	−10	Lived
					Group D—Cord cut, C; sympathectomy, S								
1 C	5.9	13.6					1; 0	20.4		*1; 11	28.4		1; 14
2 C	5.4			4; 17	17.9		7; 15	19.4		10; 45	18.1		15–17;
3 C	5.7	16.6	45	3; 20	20.2	−29	5; 19	18.6	−31	8; 55	18.9	−32	Lived
1 S		16.1		4; 53	15.1		6; 35	16.2		8; 35	15.4		Lived

All times refer to the intervals which elapsed after the commencement of trauma.

Sample 1 is the initial pre-anesthetic sample.

Sample 4 is a terminal sample in those dogs which died. All plasmas were drawn from the jugular vein except those with an asterisk which were from the carotid artery and those marked with a dagger which were cardiac samples.

The dogs in group D were either sympathectomized or had the spinal cord transected 2-6 days before the experiment.

plotted in figure 1. In order to facilitate comparison of the control values with those of the traumatized dogs it was found convenient to divide the total period of observation into the following intervals, the first 40 per cent, the second 40 per cent, and the final 20 per cent. Ether (2) and other anesthetics (7) have previously been reported to lower plasma potassium. Although no effort was made to record this fall, it is evident in some of the individual curves (fig. 1) and is sufficient to produce an average decrease of 8 per cent of the pre-anesthetic concentration during the first 40 per cent of the period studied. During the next time interval of 40 per cent the concentration returned to normal (average

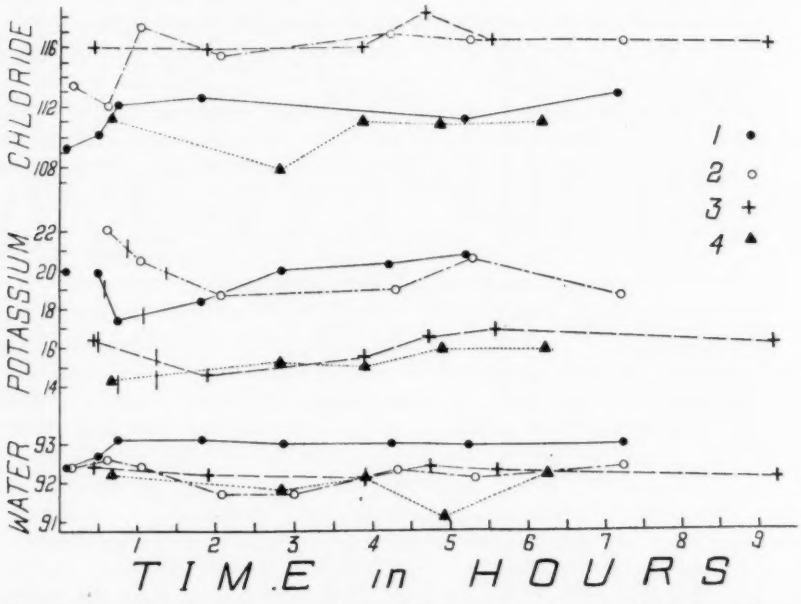


Fig. 1. The chloride (in m. eq. per l.), potassium (in mgm. per 100 grams) and water (in grams per 100 grams) in the jugular vein plasma of 4 control dogs, which were subjected to ether anesthesia for the time intervals indicated by the distance between the two upright strokes on the potassium curves.

change, 0.6 per cent decrease of the initial value) thus showing the transitory nature of the effect of anesthesia. Although the average concentration did not rise above normal during this time, it is important to record that there was one potassium increase of 10 per cent, all others remaining below 6 per cent.

(b) *Plasma K increase due to muscular trauma.* The data obtained on 27 dogs are listed in tables 1 and 2 in the order of the periods of time survived following trauma. The first series included 13 dogs, 5 of which recovered (see group C, tables 1 and 2) while 8 succumbed. From these, blood samples were taken at 1 or 2 hour intervals during the experiment. Nine of the more complete time courses are plotted in figure 2. During the first 80 per cent of its

TABLE 2

Plasma water and chloride concentrations, magnitude of leg swelling at death and remarks concerning the condition of each dog

DOG NO.	SAMPLE 1		LATER SAMPLE			LEG SWELLING	REMARKS
	H_2O	Cl	Time	ΔH_2O	ΔCl		
	Group A. Died. Terminal sample obtained						
	gm. per cent	*m.eq./l.*	*hr.; m.*	*per cent*	*per cent*	*per cent Bl. vol.*	
5	92.0	117.0	*2; 30	+0.54	−7.4	32	Slight intraduodenal hemorrhage
6	92.6	107.5	3; 45	−0.43	−5.7	71	Note hemoconcentration and leg swelling
7	92.5	102.6	5; 0	+0.75	−3.2		Vomiting. Dog in shock at 5 hours
8			6; 15	+0.32	−2.7	47	Some spreading of blood through abdominal wall
4	92.0	114.5	5; 25	−0.54	−2.0		Shock in 6 hr., B.P. = 80. In 6 hr. 26 m., B.P. = 36
9	92.4		*7; 10	+0.22	−11.4	48	Extensive spreading throughout abdominal wall
10	92.5	111.9	7; 25	−0.43	−10.2		In 5 hr. 50 m. in deep shock—B.P. = 57
11	93.0	119.5	8; 10	−0.96	−4.7	0	Slight intraduodenal hemorrhage
12	92.7	114.0	*10; 5	−0.21	−1.7	38	Marked intra-intestinal hemorrhage
13	92.6	107.9	6; 5	−1.4	−3.6	45+	Extensive spreading throughout abdominal wall
14	92.0		*17; 0	−1.3		45	In deep shock at 17 hours
	Group B. Died. No terminal sample obtained						
15	92.0	109.7	1; 35	+0.22	+2.0		Note that K was still low 2 hours before death
16	91.6		4; 30	+0.87		45	Marked hemorrhage in duodenum
17	92.4	107.3	1; 55	+1.1	+1.5	44	Did not recover from anesthetic
18	91.6	107.8	4; 48	−0.33	−6.8		
19	92.5		2; 40	0.0		48	
20			7; 15	+0.43	−8.4	46	B.P. seemed low at 7 hr. 15 m.
21	91.9	107.1	7; 35	−0.54	0.0		B.P. seemed low at 7 hr. 35 m.
	Group C. Lived						
22			8; 10		−2.1		Note that K rose, then fell
23	92.5	110.2	5; 20	+0.11	+4.3	0	No leg swelling in 7 hours
24		111.6	10; 35		−3.4		Note that K rose, then fell
25	92.0	120.6	8; 5	−0.54	−5.1		At 8 hr. 20 m. signs of physical weakness were evident
26	92.0	112.9	14; 0	−0.87	−10.0		Note that K rose although dog lived
Av.	92.2	111.5					

The interval of time listed under "later sample" is the period which had elapsed since commencement of trauma. ΔH_2O and ΔCl are expressed in per cent of the initial concentration except in a few cases when this value was not obtained; then the difference from an average value for H_2O of 92.2 per cent and for Cl of 111.5 was calculated. The asterisks refer to carotid samples; all others were from the jugular vein.

course, every curve shows a maximum rise in potassium concentration which is greater than 10 per cent of the pre-anesthetic value, this being the largest increase found in the controls. Of these maxima the smallest recorded in figure 2 was +22 per cent. It is of interest that these observations apply to dogs in which the trauma was not sufficiently severe to cause death, as well as to those which succumbed. Some of the dogs which lived may have suffered slight but not fatal shock, such as dog 25 (table 2), for example, which showed signs of weakness and prostration although it subsequently recovered. In any case a

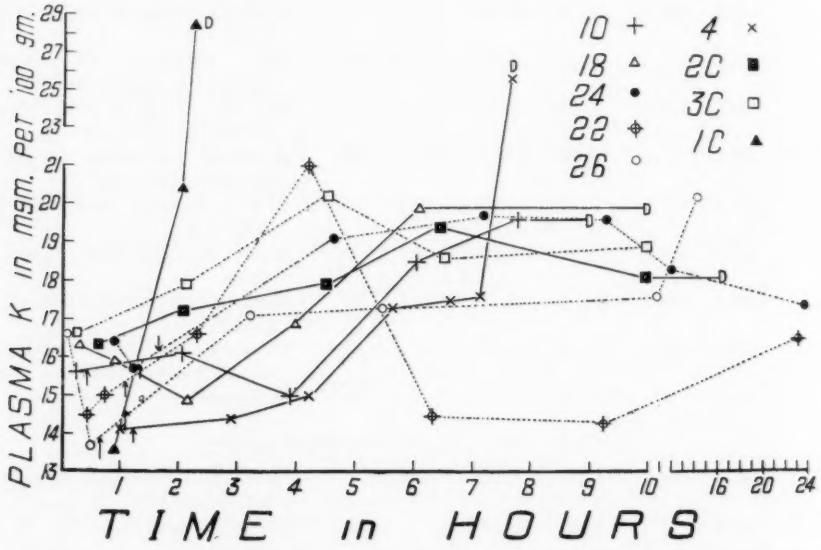


Fig. 2. The potassium concentrations in the jugular vein plasma of 9 dogs subjected to mild muscular trauma under ether anesthesia. When pre-anesthetic samples were obtained, arrows show the beginning of the traumatization which required ½ to ¾ of an hour. The 5 dogs marked with continuous lines died at the times indicated by *D*; the other 4 recovered. In dogs 2C, 3C and 1C the spinal cords were transected 2 to 6 days before the experiment.

comparison of these graphs (fig. 2) with those in figure 1 shows clearly that plasma potassium rises significantly as a consequence of muscular trauma.

In the next series considered all of the dogs died and only pre-anesthetic samples, samples taken several hours after trauma, and terminal samples were analysed. It is obvious from the character of the time courses plotted in figure 2 that this method of sampling will not always demonstrate the maximum increase in plasma potassium. Hence a considerable variation in sample 2, table 1, is to be expected. Nevertheless there were 9 cases in groups A and B where a significant rise was recorded in sample 2 and some of these were so large that an average increase of 13 per cent was obtained. Attention is drawn to group D in which it was shown that animals with spinal cords transected like-

wise exhibited an increase in the jugular vein potassium. In addition, two other dogs not listed were used for cross-circulation experiments after traumatization. The potassium increase of the traumatized donor was from 13 to 17 in one case and 15 to 29 in the other. Both uninjured recipients lived, one showing a plasma potassium change of from 15 to 12 mgm. and the other 14 to 17, the final values quoted being those found at the death of the donor.

In order to summarize the data a mass plot was made (fig. 3) of the observations on all of the dogs in the second series and on 3 of those in the first series

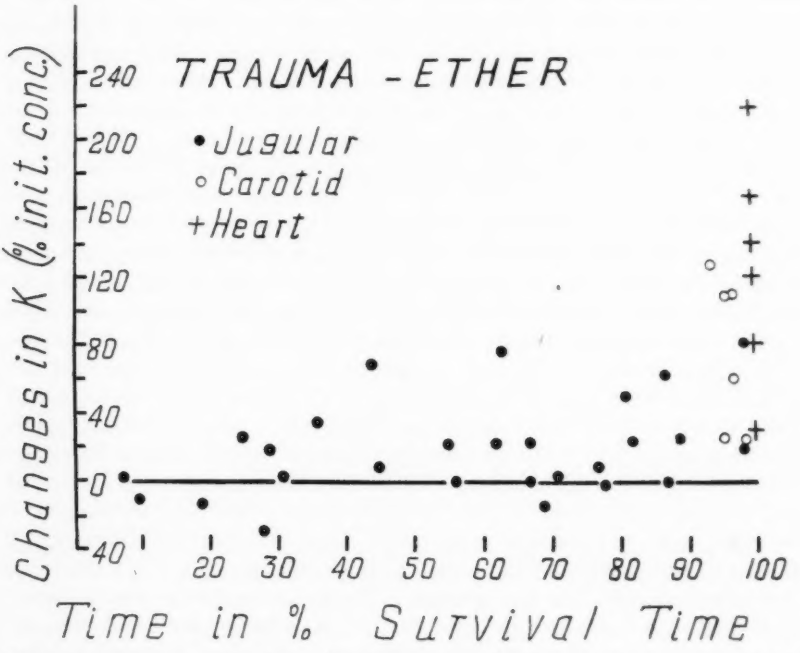


Fig. 3. A time course plot of the plasma potassium changes which occurred in 16 dogs between the commencement of muscular trauma and death due to secondary shock. Survival time is the period between the beginning of trauma and death, death being judged by the cessation of the heart beat.

which died, and for which pre-anesthetic values were obtained. The K-lowering effect of the anesthetic is evident early in the period of survival. However, the average over the first 40 per cent of the survival time was +4 per cent (p.e. ± 13 per cent) of the pre-anesthetic concentration. (Note the decrease of 8 per cent, p.e. ± 4 per cent, observed in the controls.) During the second time interval of 40 per cent the potassium rose sufficiently to give an average increase of 19 per cent (p.e. ± 18 per cent) which is considerably higher than the average decrease of 0.6 per cent (p.e. ± 4 per cent) observed in the controls and is twice the highest individual increase in the controls. Hence, although much

variation exists, the above figures illustrate that the chances are great that jugular vein plasma potassium will rise significantly as a result of trauma.

The potassium changes discussed above occurred considerably before the onset of death, but the greatest increase is evident in the last tenth of the period of survival. Although little significance is attached to this increase it cannot be attributed to the phenomenon of death alone because it was relatively small in some samples taken at death (see dogs 8, 4, 12 and 13, tables 1 and 2). The terminal samples are not strictly comparable with the earlier ones since they were taken from the carotid artery or from the heart. The reason for the increase at death is not definitely known. Dennis and Moore (5) report a large loss of potassium from the heart into the coronary veins after 5 to 9 minutes of ischemia produced by ligating the coronary arteries. Attention is drawn to dog 7 from which a heart blood sample was procured 35 minutes after death. If the terminal rise in potassium in heart plasma were due to its liberation from heart muscle because of anoxia of the tissue a much higher value than 44 mgm. per 100 ml. might be expected, since the potassium concentration in normal heart tissue is close to 400 mgm. per 100 ml. of tissue water. However, if either the liver or the heart liberated a certain fraction of its potassium (10) at some point in the progression of asphyxial symptoms at death, this would account for the fact that a maximum value seems to be reached which is not surpassed even in blood exposed to heart muscle for $\frac{1}{2}$ hour after death (dog 3).

(c) *Tissue analyses.* No matter what is the source of the terminal rise in potassium, it is reasonable to suspect that the increase earlier in the period of survival is due to liberation from the injured muscle. Some preliminary analyses of tissues and of fluid collected from the region of injury (trauma fluid) are assembled in table 3. Although no large blood vessels were severed as a result of the traumatization, dark red fluid gathered in the subcutaneous tissue. This fluid could be collected and measured. After centrifuging, the supernatant showed a lower water content, a lower chloride concentration and a much higher potassium concentration than plasma. The results suggested that plasma and some cells leaked into the area and that to the resulting fluid had been added potassium from injured muscle cells and muscle cell solids. It should be recalled that, since dog red cells have a preponderance of sodium rather than potassium, hemolysed cells will not contribute appreciably to the potassium of the fluid. In a few cases femoral arterio-venous potassium differences were determined and found to be surprisingly small. Although this indicates slow leakage of potassium into the general circulation through venous channels, it does not preclude rapid entrance via lymphatics.

The tissues analysed give some evidence of the extent of the potassium loss. Using the triceps of the forelimb as a point of reference it can be seen (table 3) that the extensors of the knee are scarcely affected by the injury to a small portion of the flexors. The gastrocnemius shows some loss of potassium and gain in water and chloride. In dog 27 a muscle bundle was found to be broken as a result of the injury. Only the broken ends lost most of their normal potassium content. Tissue taken next to these ends still retained more than half

of its normal concentration. If 100 grams of muscle lost half of its potassium about 200 mgm. of potassium would be liberated, which seems a relatively large amount, although a normal animal could easily cope with it.

2. *Plasma water and chloride.* The changes in the water and chloride concentrations of the plasma were small and will not be presented in detail. Table 2 shows the normal pre-anesthetic values (sample 1) and the maximum percentage changes. Neither water nor chloride changed appreciably during the day in the control animals plotted in figure 1, but table 2 shows that following trauma there is a small but definite decrease in chloride concentration. In no case will the dilution of the plasma account for this fall. Only terminal changes are presented in the table but the fall in chloride concentration was gradual and

TABLE 3

Tissue and fluid analyses

(All figures are expressed in units per kilogram fresh tissue except the chloride of plasma and of trauma fluid which is in m. eq. per l.)

TISSUE	DOG 7			DOG 4			DOG 1C			DOG 27		
	H_2O	Cl	K	H_2O	Cl	K	H_2O	Cl	K	H_2O	Cl	K
	gm.	*m.eq.*	*m.eq.*	*gm.*	*m.eq.*	*m.eq.*	*gm.*	*m.eq.*	*m.eq.*	*gm.*	*m.eq.*	*m.eq.*
Triceps	757	15.0	107.0	749	13.5	109.8	753	17.2	91.2	760	12.1	104.8
Extensors		27.4	92.7	757	14.0	104.0	760	15.2	100.6			
Flexors		44.0								774*	63.5*	26.1*
Flexors										780†	41.9†	60.0†
Gastroc.				771	28.1	82.3						
Trauma fluid				866	98.9	17.6					100.7	9.8
Plasma				915	116.1	6.6	915		7.3	933	112.0	5.7

Muscles analysed were triceps of the fore limb which were far removed from the site of injury, extensors of the knee from the region of injury but not themselves damaged, and the flexors of the knee which were the injured muscles. Figures with an asterisk are analyses of the cut ends of a severed muscle bundle; while those with a dagger refer to the tissue adjacent to the cut ends. Trauma fluid is the supernatant obtained by centrifuging the fluid collected from the injured area.

progressive, the greatest always being found in heart plasma. These findings are not in accord with the results of Bisgard et al. (2) who report that the plasma chloride concentration is not altered in traumatic shock. The chloride is not decreased due to excretion since oliguria persists in these dogs. It could result in part from increased CO_2 tension, from the entrance of chloride-free intracellular water into the plasma or from the exchange of chloride for some anion in the injured area.

3. *Changes in red cell volume and local fluid loss.* The consistently small changes in red cell volume (table 1) and the constant water content of the plasma suggest that whole blood and not plasma or plasma water was lost at the site of injury. The hematocrit variations show more evidence of hemodilution than of hemoconcentration, although often a slight dilution was followed by subsequent concentration. That a considerable amount of whole blood is trapped in the

injured limbs is demonstrated by the extensive hemolysis evident in the trauma fluid; all jugular plasma samples taken after trauma likewise showed signs of hemolysis. The hemodilution which is evident in some cases may result from the entrance of extracellular or intracellular water from elsewhere in the body or merely from the retention of red cells in the injured area due to the clamping down of small vessels there. Hemodilution usually accompanies the type of shock produced by bleeding from a large artery but Blalock (3) obtained hemoconcentration as a result of slow and intermittent bleeding. Measurements of the volume of the injured limbs immediately after trauma and again at death (table 4) illustrate that considerable variation in the time of bleeding into the limbs can occur. In most cases the results suggest a slow bleeding throughout the course of the experiment. Hence, fairly constant hematocrit values are to be expected since several factors probably operate simultaneously, some tending to increase and others to decrease the red cell volume.

To estimate the swelling of the injured limbs each limb was immersed, up to a certain mark, in a container of water and the volume of water displaced before and after trauma and at death was measured. The method is in error if there is extensive spreading of the hemorrhage up the abdominal wall. This occurred in only 2 cases. In other instances when the spreading was slight the affected tissue was dissected and measured. In general, as table 4 indicates, the swelling is greater at death than just following trauma, suggesting that slow bleeding into the injured area typifies this type of shock.

To evaluate the contribution to the cause of death made by local fluid loss, the amount of limb swelling in these experiments was compared to the quantities of blood removed when death results from frank hemorrhage. In the latter case it is generally agreed that a loss of 50 per cent of the calculated blood volume is fatal. The swelling of the limbs is so great, particularly since the values obtained are minimal, that hemorrhage at the site of injury must be a major factor in the cause of death. In addition the blood volume will be further decreased by the blood taken for analysis and by that lost due to intra-intestinal hemorrhage (see table 4). However, although in many cases the amount of bleeding is itself sufficient to cause death, there are also instances in which the limb swelling is slight. There seems too to be little if any relation in group 1, table 4, between the survival time and the magnitude of the limb swelling. Furthermore, in the group of dogs which died the losses in all but 3 cases were less than 50 per cent of the calculated blood volume; and, of those which lived 24 hours or longer; 3 lost more than 50 per cent of the blood volume.

Discussion. The plasma electrolyte changes in shock have been likened by Scudder and his associates to the changes in adrenal insufficiency. The latter condition is characterized by decreased chloride and sodium concentrations accompanied by hemoconcentration, an increased solid content and an increased potassium concentration in plasma. In the condition reported in this paper there is no evidence of increased solid content in the plasma, and more evidence of hemodilution than hemoconcentration. The chloride changes (av. = 4.1 m.eq.per l.), although in the same direction as in adrenal insufficiency, are much

less than in most cases reported. We did not conduct a complete study of sodium changes but in the three dogs whose plasmas were analysed the concentration did not vary appreciably or consistently from the normal although the animals died of shock. The only real similarity to adrenal insufficiency is the

TABLE 4

Relation of local fluid loss to survival time

GROUP I—DIED				GROUP II—KILLED AFTER 24–30 HOURS		
Dog no.	Limb vol. increase (per cent bl. vol.)		Survival time	Dog no.	Limb vol. increase (per cent bl. vol.)	
	1	2			1	2
			hr.; min.			
1C		28	1; 13			
28	9.7	13	1; 20	39		22
5*	32	32	2; 40	40	16	
29†	32	43	3; 0	41	7	
30	35	52	3; 0	42	20	
31	33	46	5; 0	43	11	
6	47	71	5; 35	44	16	
16†	27	45	5; 45	45	12	
8		47	6; 17	46*	34	0
9	41	48	7; 30	47*	17	30
11*	24	0	8; 35	48	0	
17		44	7–9;	49	22	
20	37	46	8–10;	50	33	54
32		37	9 app.	51	33	42
12†	17	38	10; 14	52	17	47
19	13	48	10; 50	53	38	38+
33		33	11; 15	54*	14	51
34	43	63	11; 25	55	25	57
35*	24	42	11; 40	56	23	34
36		35	15; 20	23		0
37		34	15; 0			
13		45+	17; 5			
14*	17	45	17; 35			
38	15	38	24; 0			
Average.....	28	41			20	34

Limb volume increase expressed in per cent of a calculated blood volume (bl. vol. = 8 per cent of the body weight) was measured immediately after traumatization (1), and again at death (2). Dogs from 31 to 56 were carefully examined for intestinal hemorrhage. Those marked with a dagger showed severe intra-intestinal hemorrhage, while those with an asterisk showed a slight amount.

increased plasma potassium. Even this similarity is not particularly striking since a few animals succumbed without much change in the jugular vein potassium until within a few minutes of death. A precise comparison with adrenal insufficiency is difficult since the blood vessels used for sampling by each investigator differed, and also our dogs were exposed to the K-lowering effect of

anesthesia. Furthermore, because the largest increase occurred just at death, it is important to know the proximity to death of the samples taken in adrenal insufficiency. Muntwyler et al. (9) report values of 35 mgm. per cent (range 10–74) in femoral artery plasma of 11 dogs in "rather marked insufficiency." The average value simulates ours (table 1) although the range is much greater.

There is little to be said regarding the toxic effects of potassium in experiments where the fluid loss is so great that it could be the sole cause of death. Injection of potassium salts sufficient to raise the plasma concentration to 50–60 mgm. per cent was shown to cause death by intraventricular block and cardiac arrest (13). The shocked dogs did not die for this reason because even in heart samples at or after death (table 1) the concentration did not rise to such high levels. Little is known about the toxic effects of potassium other than those on the heart. Hence, although it is unlikely that potassium caused death, the concentration becomes abnormally high and it might indeed be a contributing factor.

The data presented here show clearly that a significant increase in jugular vein potassium follows muscular trauma, but there is some variation from dog to dog. This variability is to be expected if one accepts as a tentative working hypothesis the following sequence of events: that ether causes a transitory lowering of plasma potassium, that the injured muscle liberates potassium which is not excreted because of the oliguria which persists, that the tissues probably absorb as much potassium as they can under the conditions of the experiment.

SUMMARY

The blood of twenty-seven dogs was studied after the dogs had been subjected to mild trauma of the muscles under ether anesthesia. They succumbed in secondary shock in 3 to 17 hours after the commencement of the traumization. The condition of shock is characterized by considerable swelling in the injured regions, by a slight decrease or no change in the red cell concentration, and by little alteration in the chloride or water concentration of the jugular vein plasma. A small but significant increase in plasma potassium occurs considerably before death, and an increase of 100 to 200 per cent at or just prior to death. That local fluid loss was the major factor in the cause of death was concluded from a larger series of animals in which the swelling of each traumatized limb was measured.

Grateful acknowledgment is made of the receipt of financial assistance from the Best Medical Research Fund to one of us (J. F. M.). We wish also to express our indebtedness to Mr. C. Cowan for valuable assistance throughout this research.

REFERENCES

(1) Best, C. H. and D. Y. Solandt. Can. Med. Assoc. J. **43**: 206, 1940.
(2) Bisgard, J. D., A. R. McIntyre and W. Asheroff. Surgery **4**: 528, 1938.
(3) Blalock, A. Principles of surgical care. Shock and other problems. Mosby, St. Louis, 1940.

(4) Butler, A. M. and E. Tuthill. J. Biol. Chem. **93:** 171, 1931.
(5) Dennis, J. and R. M. Moore. This Journal **123:** 443, 1938.
(6) Fenn, W. O., D. M. Cobb, J. F. Manery and W. R. Bloor. This Journal **121:** 595, 1938.
(7) Fenn, W. O. Physiol. Rev. **20:** 377, 1940.
(8) Manery, J. F., I. S. Danielson and A. B. Hastings. J. Biol. Chem. **124:** 359, 1938.
(9) Muntwyler, E., R. C. Mellors and F. R. Mautz. J. Biol. Chem. **134:** 345, 1940.
(10) Noonan, T. R., W. O. Fenn and L. Haege. This Journal **132:** 474, 1941.
(11) Pen, D. F., J. Campbell and J. F. Manery. Unpublished.
(12) Scudder, J. Shock: Blood studies as a guide to therapy. J. B. Lippincott Co., Philadelphia, 1940.
(13) Winkler, A. W., H. E. Hoff and P. K. Smith. This Journal **127:** 430, 1939.
(14) Zwemer, R. L. and J. Scudder. Surgery **4:** 510, 1938.

THE RÔLE OF OXYGEN IN THE METABOLISM AND MOTILITY OF HUMAN SPERMATOZOA

JOHN MACLEOD[1]

From the Department of Anatomy, Cornell University Medical College

Received for publication September 11, 1942

The experiments reported here are a continuation of the work on human spermatozoa which was begun with the object of studying the metabolic behavior of these cells. In previous papers (7, 8), the metabolism was shown to be almost exclusively glycolytic, the oxygen consumption being of such small magnitude that it could not properly be interpreted as a true respiration. Furthermore, no spectroscopic evidence of cytochrome could be found and it was observed that, in certain cases, motility was profoundly depressed in the presence of pure oxygen. These observations have been confirmed in every essential respect by Ross et al. (12) except that their mean figures for glycolysis are higher than those given by this author (7). Since the latter results were published, higher and more consistent figures[2] have been obtained from the combined spermatozoa of a small new group of donors. These figures are in closer agreement with those of Ross et al. and show, furthermore, that the level of aerobic glycolysis is virtually the same as that of the anaerobic.

The present extension of these studies was undertaken to determine more precisely the behavior of the spermatozoa under aerobic conditions and particularly to examine the link between glycolysis and respiration in these cells. The experiments entailed 1, determination of the activity of certain dehydrogenase systems; 2, the measurement of oxygen consumption in the presence of different substrates, and 3, testing for the presence of the cytochrome complex by indirect methods. Lastly, the toxic effect of high oxygen tensions was investigated from the point of view of a possible formation of hydrogen peroxide by the spermatozoa.

METHODS. The spermatozoa suspensions were prepared by methods described previously (7, 8). For the measurement of oxygen consumption, the cells were suspended in glucose-free Ringer-phosphate solution (pH 7.35) and shaken in Warburg manometers at 38°C. Various substrates were added directly to the cell suspensions in the Warburg vessels prior to temperature equilibration. The final concentration of each substrate was M/100. Cell motility was determined before and after every experiment by methods already

[1] Aided by a grant from the National Committee on Maternal Health.

[2] The new figures are as follows:

No. of expts.	60
$mm^3CO_2/10^8$cells/hr. in N_2	25
$mm^3CO_2/10^8$cells/hr. in O_2	24

It should be stated, however, that figures as high as these were obtained previously from some individual specimens (7).

described (8). For the determination of cytochrome and cytochrome oxidase activity, the p-phenylene diamine was added to the cell suspensions according to the techniques outlined by Keilin and Hartree (5) and Stotz et al. (13). These experiments will be described in detail below.

A modified Thunberg technique, which allowed simultaneous measurement of glycolysis and methylene blue reduction, was used to measure the dehydrogenase activity of the spermatozoa in the presence of certain substrates. The cell suspensions were prepared for the measurement of glycolysis (0.03M bicarbonate being substituted for phosphate) and equilibrated for 20 minutes at 38°C. with 95 per cent N_2—5 per cent CO_2. Methylene blue was placed in the side-arms of the Warburg vessels and added to the suspensions in the main vessels when equilibration was complete. Thereafter, at intervals, the manometers were removed temporarily from the water-bath to determine when reduction of the dye was complete.

EXPERIMENTS. The dehydrogenases of the spermatozoa were investigated using a variety of substrates which included glucose, succinate, pyruvate, fumarate and lactate among others. Only in the presence of glucose and succinate was any rapid or marked reduction of the methylene blue seen.[3] The fastest reduction times were seen invariably in the presence of glucose and to a lesser degree when succinate was present. In the absence of substrate or in the presence of the other substrates mentioned above no marked reduction of the dye took place over a period of many hours. These results are in marked contrast to those of Lardy and Phillips (6) who showed that the reduction of methylene blue by bull spermatozoa took place rapidly in the *absence* of substrate and in the presence of succinate and fumarate but was inhibited when glucose was added.

The importance of the above results for human spermatozoa lies not so much in the reduction time values but in the active reduction in the presence of succinate. This was the first positive evidence obtained of the presence in the cells of the succinic dehydrogenase and, therefore, of the probable presence of the cytochrome system (2). This evidence was amplified further when malonate (M/100), a known inhibitor of the succinic dehydrogenase (11), was added to the spermatozoa suspensions reducing the dye in the presence of succinate. Malonate almost completely inhibits this reduction but has no effect on the reduction time in the presence of glucose.

Effect of succinate on the oxygen consumption. These results suggested a re-examination of the oxygen consumption of the spermatozoa particularly in relation to the presence of the cytochrome complex. It seems definitely established (2) that the succinic dehydrogenase is, structurally and chemically, intimately linked with the cytochrome system. When the dehydrogenase is reduced, it is oxidized by cytochrome C almost instantaneously (4) and not to any appreciable

[3] There are certain objections to the use of intact cells for the measurement of enzyme activity, mainly that of the possible difference in penetration of the intact cell of different substrates. The figures given in table 1 serve merely to indicate that enzymatic activity towards a particular substrate is present and are not intended to demonstrate the *maximal* activity toward that substrate.

extent by molecular oxygen. As shown above, methylene blue can also act as the hydrogen acceptor. Therefore, it seemed probable that, if succinate was substituted as a substrate for glucose, the oxygen consumption of the spermatozoa would be increased. This proved to be the case. The figures in table 1 show the results obtained when oxygen consumption was measured in the absence of substrate and in the presence of glucose and succinate.

The figures obtained in the presence of glucose are similar to those reported previously (7). The increase in oxygen consumption in the absence of substrate, while small, is significant since it appeared in every experiment. The inhibiting effect of glucose has also been noted in ejaculated bull spermatozoa (6) but not in cells obtained from the epididymis (3). However, in the presence of succinate the human cells show a relatively high and stable oxygen consump-

TABLE 1

Oxygen consumption of human spermatozoa in the presence of different substrates

NO. OF EXPTS.	NO. SUBSTRATE	GLUCOSE	SUCCINATE	p-PHENYLENE DIAMINE (M/50)
20	1.43*	1.34	6.3	18

* The figures represent the oxygen consumption per $mm^3/10^8$cells/hour.

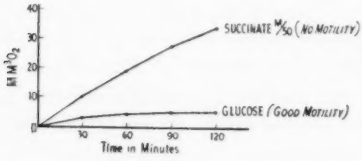


Fig. 1

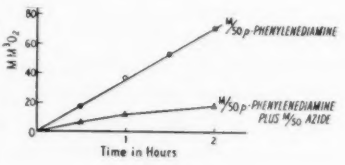


Fig. 2

Fig. 1. The oxygen consumption and motility of human spermatozoa in the presence of glucose and sodium succinate.

Fig. 2. The oxidation of p-phenylenediamine by human spermatozoa.

tion (fig. 1). In every case it was evident that the oxidation of succinate was vigorous and progressive compared to that of glucose. The addition of malonate or of azide inhibits the succinate oxidation to the autoxidation level. This evidence supplements that given above for the inhibition of methylene blue reduction by malonate in the presence of succinate. It also complements the observations of other authors (2, 4) which show the oxidation of succinate to be mediated by the azide-sensitive cytochrome complex.

Neither fumarate, lactate or pyruvate increases the oxygen consumption above the autoxidation level. The failure of the spermatozoa to oxidize lactate is to be expected since they produce as much lactic acid in oxygen as they do in nitrogen. Their failure to oxidize fumarate, on the other hand, suggests that the oxidation of succinate is a one-step process, namely, the removal of hydrogen to form fumaric acid and the subsequent accumulation of the latter compound.

In spite of the vigorous oxidation of succinate by the spermatozoa, any energy made available in this reaction is not coupled with motile activity. In *all* experiments in which succinate was substituted for glucose, the motility failed as rapidly as if no substrate was present.

The oxidation of p-phenylenediamine. As a further check on the presence of the cytochromes and cytochrome oxidase in the spermatozoa, the ability of the cells to oxidize p-phenylenediamine was determined. Preliminary experiments showed that the autoxidation of this substance was negligible but that it was rapidly oxidized by the spermatozoa. The concentration necessary to produce maximal oxidation was M/50. The results are shown in table 1.

Keilin and Hartree (5) and Stotz et al. (13) have shown that the oxidation of p-phenylenediamine is mediated through cytochrome C and cytochrome oxidase and that its oxidation can be used as an indicator of the presence of such a system in cells. These authors have shown further that cytochrome B can be oxidized by molecular oxygen, but is insensitive to the inhibitory effect of cyanide and azide, and therefore can act independently of cytochrome oxidase. Stotz et al. (13) demonstrated a relatively cyanide-insensitive oxidation of p-phenylenediamine and showed conclusively that the remaining catalysis was due to cytochrome B. In the case of human spermatozoa approximately 20 per cent of the oxidation of p-phenylenediamine escapes azide inhibition (fig. 2). In line with the evidence of Stotz et al., this can be attributed to the presence of cytochrome B.

In view of the evidence given above, it is reasonable to conclude that in spite of the very small oxygen consumption of human spermatozoa, these cells contain a virtually complete respiratory system, namely, succinic dehydrogenase, cytochromes B and C and cytochrome oxidase,[4] and are capable of carrying on oxidative processes if the appropriate substrate is present.

The effect of high oxygen tensions on motility. Any analysis of the rôle of oxygen in the metabolism and motility of human spermatozoa would not be complete without a consideration of the phenomenon first described by this author (7) and confirmed by Ross et al. (12), namely, that of the loss of motility which often occurs when these cells are exposed to oxygen at 38°C. for several hours (table 2). It has already been shown that the spermatozoa will retain maximal motility in nitrogen at 38°C. for many hours (7). This evidence, coupled with the low oxygen consumption of the cells and their ability to get all their motile energy from a process which involved the breakdown of glucose to lactic acid, suggested that the toxicity of oxygen may be related to a similar process which retards the growth of certain anaerobic bacteria in air. MacLeod and Gordon (9) have shown that certain of these bacteria under aerobic conditions produce

[4] The absolute amount of these substances in the spermatozoa is exceedingly difficult to determine. Only a few milligrams of tissue (wet) are available for any given experiment and, since isolation of enzymes requires relatively large amounts of fresh material, certain extensions of this work on human spermatozoa are severely limited. As would be expected, the addition of cytochrome C to the intact cells in the presence of p-phenylenediamine causes no increment in the oxygen consumption.

enough hydrogen peroxide in the course of their metabolism either to destroy the growing colony or prevent its multiplication. Accordingly, experiments were designed to determine whether a similar phenomenon in human spermatozoa might be responsible for the loss of motility in high oxygen tensions. Two spermatozoa suspensions were set up in the usual way for measurement of aerobic glycolysis and equilibrated with 95 per cent O_2—5 per cent CO_2 for 15 minutes at 38°C. In similar fashion, another two suspensions of the same spermatozoa were set up, except that 0.1 cc. of dilute hemoglobin or catalase[5] was added to each on the assumption that if any hydrogen peroxide was produced in the system, it would be destroyed instantaneously either by the peroxidase activity of the hemoglobin or the catalatic activity of the catalase. The manometers containing the spermatozoa exposed to oxygen were then run at 38°C. for periods up to 9 hours and the motility examined at the end of the experiment. Typical experiments are shown in table 2. In every case where loss of motility oc-

TABLE 2

Spontaneous loss of motility in 95 per cent oxygen and the protective effect of catalase

EXPT. TIME	ACTIVITY OF SPERMATOZOA AT END OF EXPERIMENT		
	95% nitrogen—5% CO_2	95% O_2—5% CO_2	95% O_2—5% CO_2 and Catalase
hours			
8	4 (58%)	No motility	4 (55%)
8	3+ (50%)	No motility	3+ (52%)
7	4 (60%)	No motility	4 (50%)
9	3 (50%)	No motility	3 (49%)
5	3+ (32%)	1 (10%)	3+ (32%)
5	4 (68%)	2 (10%)	4 (70%)

The figures in parentheses denote the number of motile cells. The other figures denote the quality of motility, 4 being maximal motility.

curred in the presence of oxygen, the addition of hemoglobin or of catalase was sufficient to maintain a maximal motility comparable to that of the same spermatozoa under anaerobic conditions.

These results were indicative of the production of hydrogen peroxide by the spermatozoa. It followed, therefore, that its formation and the resulting toxicity must be within the limits of the small oxygen consumption of the spermatozoa. This assumption was tested by adding amounts of hydrogen peroxide with varying oxygen equivalents to spermatozoa at 38°C. and determining its effect on motility. It was found that a concentration of peroxide[6] equivalent to between 10 and 20 mm.^{3} of oxygen was sufficient to destroy the motility of 200 million spermatozoa within 5 minutes. These oxygen equivalents are within

[5] I am indebted to Dr. Kurt G. Stern of Yale University for a generous sample of catalase of known purity.

[6] The oxygen equivalent of peroxide was determined by adding catalase to measured amounts of peroxide at 38°C. and measuring manometrically the oxygen produced.

the range of oxygen consumption which might be expected of the spermatozoa over a period of several hours. These experiments serve to demonstrate the sensitivity of the spermatozoa towards low concentrations of peroxide and indicate the nature of the chemical mechanism for the loss of motility which occurs when the cells are exposed to high oxygen tensions. Lardy and Phillips (6) have observed that bull spermatozoa show a similar sensitivity towards hydrogen peroxide.

Further investigation of the effect of oxygen on spermatozoa motility is in progress and preliminary results show that at low oxygen tensions (between 5 and 10 per cent) the toxicity is eliminated.

Discussion. The results presented here and those published previously (7, 8) indicate that oxygen is not of primary importance in the metabolism of human spermatozoa and is not essential for the maintenance of motility. On the other hand, the presence in these cells of a more or less complete respiratory system would suggest 1, that they once possessed a high respiratory activity, or 2, that their ultimate function required oxidative activity. Recent work on the metabolism of bovine epididymal spermatozoa (3) shows that the sperm in the epididymis rapidly oxidize glucose but, when ejaculated, they not only fail to oxidize glucose but the respiration is actually inhibited by this substance (3, 6). It is possible that a similar metabolic change takes place in human spermatozoa in passage from the epididymis to the seminal fluid.

In view of the rate of p-phenylenediamine oxidation, the cytochrome system is potentially capable of carrying a large oxygen consumption. That it does not do so, except in the oxidation of succinate, cannot be explained at present although it is obvious that the link between glycolysis and respiration has been broken or perhaps never existed. The spermatozoa cannot oxidize lactate or pyruvate and there is no evidence of the Pasteur effect. In the latter respect, the metabolism of human spermatozoa is, like that of jejunal mucosa (11), unique among mammalian tissues.

The conclusion has already been made (8) that human sperm derive enough energy for motility from the breakdown of glucose to lactic acid. This conclusion receives additional support from the evidence given here that the oxidation of succinate, presumably an energy-yielding reaction, does not maintain motility in the absence of glucose.

The failure of motility which often appears at 38°C. in high oxygen tensions and which can be prevented by catalase (or peroxidase) points definitely to the production of hydrogen peroxide in the system. The evidence is only indirect since the peroxide cannot be detected chemically. But the small amount of peroxide necessary to destroy motility is not easily susceptible of chemical analysis if, indeed, it exists as such for any length of time. That peroxide should be produced at all in the course of the metabolism of the spermatozoa is a matter of considerable interest since no formation of this substance has as yet been demonstrated in the cells of higher organisms (10). But such cells invariably have an active respiration and contain enough catalase rapidly to destroy any peroxide which may be formed (10). The virtual absence of respiration in the

spermatozoa and their pronounced sensitivity towards peroxide poisoning suggests a deficiency of catalase in these cells.

Finding the source of the peroxide in the metabolism of the spermatozoa awaits further analysis of the enzyme systems. Autoxidation of a flavoprotein is a strong possibility since hydrogen peroxide is the end-product of such a reaction. Theorell (14, 15) has shown that the reoxidation of flavoprotein is normally accomplished by the terminal respiratory system (cytochrome C) but if it cannot do so, it will react directly with molecular oxygen, particularly at *high oxygen pressures*. Since the cytochrome system in the spermatozoa is so inactive and since the possible peroxide formation takes place only at high oxygen pressure, the mechanism suggested above is tenable.

SUMMARY

1. The rôle of oxygen is not of primary importance in the metabolism and motility of human spermatozoa.

2. These cells possess a complete terminal respiratory system but can not oxidize glucose or its anaerobic breakdown products, lactate and pyruvate.

3. Succinic acid is oxidized but this reaction is not coupled with motility in the sense that any energy made available can be used for motile activity.

4. In regard to the depressing effect of high oxygen pressure on the motility, evidence is presented suggesting the production of small amounts of hydrogen peroxide by the spermatozoa and a possible mechanism for its production is discussed.

REFERENCES

(1) DICKENS, F. AND H. WEIL-MALHERBE. Biochem. J. **35:** 7, 1941.
(2) GREEN, D. Mechanism of biological oxidations. Cambridge University Press, 1940.
(3) HENLE, G. AND C. A. ZITTLE. This Journal **136:** 70, 1942.
(4) KEILIN, D. Proc. Roy. Soc. London, Series B **104:** 206, 1929.
(5) KEILIN, D. AND E. F. HARTREE. Ibid. **125:** 171, 1938.
(6) LARDY, H. A. AND P. H. PHILLIPS. J. Biol. Chem. **138:** 195, 1941.
(7) MACLEOD, J. This Journal **132:** 193, 1941.
(8) MACLEOD, J. Endocrinol. **29:** 583, 1941.
(9) MACLEOD, J. W. AND J. GORDON. J. Path. and Bact. **25:** 139, 1922.
(10) OPPENHEIMER, C. AND K. G. STERN. Biological oxidation. Nordemann Publishing Co., Inc., New York, 1939.
(11) QUASTEL, J. H. Biochem. J. **19:** 304, 1925.
(12) ROSS, V., E. G. MILLAR AND R. KURZROK. Endocrinol. **28:** 885, 1941.
(13) STOTZ, E., A. E. SIDWELL, JR. AND T. R. HOGNESS. J. Biol. Chem. **124:** 733, 1938.
(14) THEORELL, H. Biochem. Ztschr. **285:** 207, 1936.
(15) THEORELL, H. Ibid. **288:** 317, 1936.

OBSERVATION ON THE VARIOUS FACTORS INFLUENCING THE INCREASE OF ERYTHROCYTIC FRAGILITY INDUCED BY STASIS[1]

CHIAO TSAI, C. J. CHEN AND K. Y. CHIU

From the Departments of Physiology and Pharmacology, College of Medicine, National Central University, Chengtu, China

Received for publication October 5, 1942

That erythrocytic fragility is increased by stasis of the blood *in vivo* and *in vitro* has been demonstrated by Waller (1939), Tsai, Lee and Wu (1940) and Ham and Castle (1940). Tsai, Lee and Wu have shown that this phenomenon is not related to pH change nor to the lowering of O_2 tension and increase of CO_2 partial pressure in the stagnant blood. In a personal communication Prof. W. B. Castle has expressed his view that the increase of fragility is not due to an effect on the membrane, but rather to an increase of osmotically active material within the red cell. The present investigation is an attempt to test the above suggestion and to study various other factors responsible for the increased fragility induced by artificial stasis.

METHODS. Dog's blood was used in all experiments. It was obtained aseptically by venipuncture. The blood after being withdrawn was immediately divided into 4 to 6 samples. One sample was used at once for fragility and lactic acid determinations, while the rest were kept in a water bath at 37°C. for varying periods from 1 to 20 hours before the tests. In prolonged experiments all the blood containers were sterilized beforehand.

The erythrocytic fragility toward hypotonic buffered saline was determined according to the method of Creed as modified by Tsai, Lee and Wu (1940). The degree of fragility was expressed in terms of percentage concentration of sodium chloride in the buffered solution that caused 50 per cent hemolysis. This was called mean corpuscular fragility (M.C.F.) by Creed (1938) and this term will be adopted in the present paper. It was obtained graphically by interpolating the percentage values of hemolysis.

The lactic acid content of the whole blood, of the cells and of the plasma was estimated by the method of Friedeman, Shaffer and Cotonio as described by Peters and Van Slyke (1931). In some experiments the blood cells were washed thrice with isotonic phosphate buffered saline (pH 7.25) and then made up with the same fluid to the original volume of the blood. They were subjected to artificial stasis as the whole blood. Since it was found that the washed cells did not exhibit increasing fragility with stasis, the lactic acid content of the cell suspensions with and without previous addition of glucose was also determined.

In another series of experiments the blood was made hypertonic by adding

[1] Preliminary reports of the present work have been published in abstract form in Proceedings of Chinese Physiological Society, Chengtu Branch, October, 1941 and February, 1942.

excessive glucose or citrate before starting artificial stasis. As this was shown to prevent the increase of fragility of the stagnant blood *in vitro*, several experiments were performed in which the stasis of blood was produced within certain organs. In two experiments with luminalized dogs we used two kidneys and two hind limbs as the stagnant organs; one side served as control, while the other side was injected with 5 to 9 ml. of 5 to 10 per cent sodium citrate or 5 to 8 ml. of 10 per cent glucose solution. In another experiment the spleen was employed in place of the legs. The viscus was tied into two approximately equal halves. In all these three experiments the organs were occluded by first ligaturing all the veins and next the arteries. Immediately after the occlusion of all the vessels a control blood sample was removed from the organ for fragility test. This was followed by an intra-arterial injection of glucose and citrate respectively into one kidney or one limb or a separated half of the spleen. Samples of the stagnant blood from these organs with and without previous glucose or citrate injection were removed after varying intervals of 2 to 5 hours and estimated for osmotic resistance as usual. Care was taken to check the colorimetric value of each sample against a common standard and due corrections were made for the percentage reading of hemolysis accordingly. Control tests regarding the possible influence of the hypertonic blood on the tonicity of the testing buffered saline were also performed and showed that the amount of the hypertonic blood used (0.02 ml.) was too small to produce noticeable alteration of the tonicity of the saline (2 ml.).

Results. *Relation to lactic acid content of the blood.* When the blood was kept *in vitro* at body temperature for varying periods of time, erythrocytic fragility increased with lactic acid concentration of the whole blood. Table 1 records the results of a typical experiment for illustration. Although this increase is not exactly parallel, our calculation based upon 15 experiments has shown that the correlation coefficient of these two variables reaches nearly 0.5. However, this may be a coincidence and does not give a convincing proof of their causative relation.

If the increase of lactic acid in the stagnant blood is the cause of increasing fragility, we should expect to find no alteration of corpuscular resistance by preventing the formation of lactic acid with sodium fluoride, and a more marked increase by previous addition of lactic acid to the blood. Regarding the first point, our experiments were unsuccessful because sodium fluoride itself lowered the osmotic resistance of the red cells. The evidence regarding the second point is also equivocal because the increase was much smaller than it should be if lactic acid accumulation were the sole cause of increased fragility. The results from a typical experiment given in table 2 suffice to clarify our contention.

The effect of washing. In the washed cells the increase of fragility by artificial stasis was not observed. This is illustrated by the data from a typical experiment given in table 3. It was usually observed that after a short time of incubation the fragility drops to a lower level and tends to rise only very slightly during the next two hours; it remains slightly below the original level even after 5 to 10 hours.

This remarkable phenomenon was not altered by adding glucose to the cell suspension (table 4), or replacing the buffered saline with old plasma standing at room temperature over 10 hours (table 5). On the other hand, once the fragility has been rendered high by prolonged stasis of the whole blood, subsequent washing of the cells cannot prevent the further fall of osmotic resistance by re-subjecting to incubation (table 5).

TABLE 1

Relation of increased fragility to lactic acid concentration

	TIME (MINUTES)				
	0	105	165	420	545
M.C.F., NaCl gram per cent	0.273	0.281	0.288	0.315	0.324
Lactic acid mgm. per cent	4.93	5.30	21.6	33.1	36.9

TABLE 2

The effect of adding lactic acid to the blood before subjecting to stasis

	DURATION OF STASIS (HOURS)				
	Immediately	4.5 hours later			
	Lactic acid added (mgm. per cent)				
	0	0	20	30	40
M.C.F., NaCl gram per cent	0.258	0.276	0.278	0.284	0.287
Lactic acid found mgm. per cent	17.0	20.8	43.0	45.0	50.6

TABLE 3

Influence of washing on fragility

DURATION OF STASIS	M.C.F. IN NaCl GRAM/100 ML.	
	Whole blood	Washed blood
minutes		
0	0.335	0.338
90	0.337	0.320
240	0.348	0.326
300	0.354	0.328

Lactic acid formation in the washed cells. From the data presented in the preceding sections it appears that some substance responsible for the increased fragility induced by stasis may have been removed by washing. In order to ascertain whether glycolysis stopped after washing, we have carried out some experiments in which the formation of lactic acid in the unwashed and washed cells was determined. As shown in table 6, lactic acid formation practically ceases after washing. However, glycolysis is not a prerequisite for the increased fragility because by adding glucose to the cell suspension the formation of lactic

acid resumes without being accompanied by the lowering of osmotic resistance (see table 4).

The effect of adding or injecting glucose and citrate. If the increased fragility induced by stasis is due to the accumulation of osmotically active substance within the cell which causes a higher osmotic pressure in the cell interior than its

TABLE 4

The effect of adding glucose to the cell suspension on the M.C.F. (results of two typical experiments)

DURATION OF STASIS	CONTROL	1 MGM. GLUCOSE ADDED TO 1 ML.		CONTROL	2 MGM. GLUCOSE ADDED TO 1 ML.	
	M.C.F.	M.C.F.	Lactic acid mgm./100 ml.	M.C.F.	M.C.F.	Lactic acid mgm./100 ml.
hours						
0	0.329	0.326	9.0	0.313	0.315	7.2
3	0.332	0.332	16.7	0.306	0.308	17.8
6	0.332	0.336	22.5	0.307	0.303	34.4

TABLE 5

The influence of old plasma on the fragility of the washed cells (results of a typical experiment)

DURATION OF STASIS	M.C.F. IN NaCl GRAM/100 ML.	
	Fresh cell + old plasma	Old cell + old plasma
hours		
0	0.304	0.319
4.5	0.300	0.318
5.5	0.301	0.338
6.5	0.306	0.343

TABLE 6

Lactic acid formation in the washed cells

UNWASHED CELLS		WASHED CELLS	
Duration of stasis	Lactic acid mgm./100 ml.	Duration of stasis	Lactic acid mgm./100 ml.
hours		*hours*	
0	12.8	2.5	8.23
2	20.6	4.5	9.02
5	24.7	6.5	9.76
7	28.8	23.0	9.25

environment, an artificial increase of osmotic pressure in the latter will balance that in the former and hence prevent the unequal migration of water molecules into the cell. With this as a working hypothesis, a number of experiments have been performed in which the cell environment was made hypertonic by adding glucose and citrate to the blood before subjecting to stasis. If the increase of osmotic pressure in the cell interior be the cause of increased fragility,

addition of slowly diffusible substance such as glucose or citrate would protect the cell.

The results from both *in vitro* and *in vivo* experiments in this series are concordant in demonstrating the fact that the increased fragility can be prevented by adding or injecting glucose or citrate to the blood before stasis. For this effect to manifest itself clearly, the glucose or citrate concentration in the blood must be high. In our *in vitro* experiments we have found that 0.5 per cent glucose or citrate in the blood produces only a very feeble effect, or in some cases no effect at all, but marked effect was observed in all cases when the final concentration reaches 2.0 per cent. In the *in vivo* experiments we have not determined the final concentration of glucose and citrate in the stagnant blood. In tables 7 and 8 are given some typical results.

TABLE 7

Influence of adding glucose and citrate to the oxalate blood before stasis

	M.C.F. IN NaCl GRAM/100 ML.		
	Duration of stasis (minutes)		
	0	180	405
Control, containing 0.2 per cent oxalate	0.347	0.350	0.357
Glucose added up to 0.5 per cent	0.344	0.344	0.354
Glucose added up to 2.0 per cent	0.343	0.343	0.334
Sodium citrate added up to 0.5 per cent	0.342	0.344	0.348
Sodium citrate added up to 2.0 per cent	0.339	0.317	0.327

TABLE 8

The effect of injecting glucose and citrate into the stagnant organs

	M.C.F. IN NaCl GRAM/100 ML.		
	Duration of stasis (minutes)		
	0	120	240
Spleen, rostral part, control	0.321	0.346	0.372
Spleen, caudal part, 9 ml. 5 per cent citrate injected	0.321	0.326	0.320
Right kidney, control	0.321	0.328	0.414
Left kidney, 8 ml. 10 per cent glucose injected	0.322		0.305

Mean corpuscular volume. Another approach to the problem is to see whether the mean corpuscular volume changes under the condition of stasis. One should expect to observe an expansion of cell volume in the stale blood if there is an unequal diffusion of water in an inward direction as a consequence of an increase of osmotic concentration within the cell. Our results confirm this deduction.

The hematocrit value and r. b. c. count of the stale oxalate (0.2 per cent) blood with and without previously adding glucose were determined in two

experiments and the mean corpuscular volume (M.C.V.) was calculated according to the conventional method. The determination of the cell volume per cent was carried out by means of the modified Van Allen hematocrit tube as described by Wu and Tsai (1940). The results of these two experiments consistently showed an increase of M.C.V. with the advance of stasis. However, it did not occur, if glucose was added up to 2 per cent before stasis treatment; as a matter of fact, the cell as revealed by microscopic examination shrank under this condition. The data given in table 9 suffice to establish the above points.

TABLE 9

The influence of stasis on the mean corpuscular volume

DURATION OF STASIS	R.B.C. (MIL./CMM.)		CELL VOLUME (PER CENT)		M.C.V. (μ^3)	
	Control	Glucose added	Control	Glucose added	Control	Glucose added
hours						
0	6.35	6.20	46	47	72.3	75.3
3	6.25	6.45	48	44	76.7	68.2
6	5.98	6.57	53	43	88.7	65.5

Discussion. Bergenhem and Fahraeus (1936) claimed to have demonstrated the presence of lysolecithin in the defibrinated or citrated blood kept for several hours at body temperature. They believed that spherocytosis in the stale blood is due to the adsorption of lysolecithin on the cell surface. Since spherocytosis is always a concomitant phenomenon of increased fragility and a predisposing condition of hemolysis, one may postulate the decreased osmotic resistance of the stale blood as a consequence of lysolecithin formation in the plasma. However, this assumption is not substantiated by our present finding because we have demonstrated that the old plasma, which, according to the above view, should contain ample lysolecithin, does not increase the fragility of the fresh washed cells subjected to stasis.

From the results described in the present report, it appears very likely that the increased fragility of the red cells induced by stasis is due to the accumulation of osmotically active metabolites in the interior of the cell which causes a transference of water from the plasma into the cell and thereby makes the cell enlarge and become more spherical, leading to increased fragility and hemolysis. In a number of experiments where the plasma and the packed cells were kept separately, we have shown that the rate of lactic acid formation in the cells during stasis is definitely faster than in the plasma. The results of one of such experiments is presented graphically in figure 1. This seems to furnish strong evidence supporting the assumption regarding the accumulation of metabolites within the cell. One must of course bear in mind that lactic acid is not the only one of the metabolic products that increases the osmotic concentration in the cell interior. Although we did not study the formation of other osmotically active substances there is every reason to believe that they would exert a similar effect.

The favorable action of washing may be explained by two alternative theories. 1. It may be ascribed to the removal of glucose and other metabolic substances whose breakdown results in an increase of osmotic concentration within the cell. Addition of glucose to the washed cell suspension may start glycolysis again, but the subsequent change of osmotic condition within the cell is counterbalanced by the glucose and its breakdown products in the cell environment whereby the increase of fragility is hindered. 2. The process of washing may in some way make the cell membrane more permeable to osmotically active substances so as to set free the cell from the accumulation of them. This explanation accords with the fact that the reintroduction of glucose into the cell suspension does not cause the cell to return to its former condition of being susceptible to stasis effect. On the same assumption one may explain the deprivation of the old

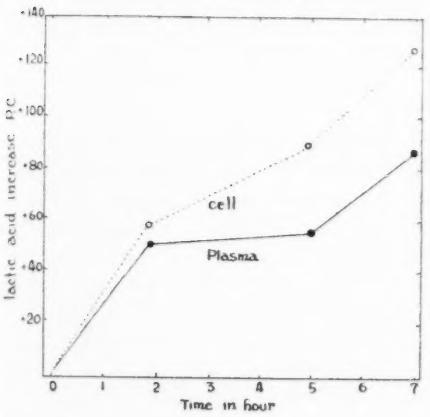


Fig. 1

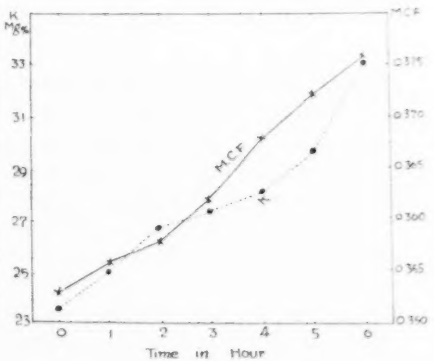


Fig. 2

Fig. 1. Showing the relative rate of lactic acid formation in the plasma and the cell during stasis.

Fig. 2. Increase of plasma K-content during stasis of the blood.

fragile cell from the ameliorating effect of washing as being due to the loss of changeability of membrane property.

The above discussion immediately leads us to the consideration of the possibility of alteration of membrane permeability during stasis. The question is far more complicated than appears at first because a change of permeability to one substance does not necessarily mean a change of permeability to other substances. We have carried out several experiments in which the plasma potassium of the blood after varying periods of stasis was estimated according to the method of Kramer and Tisdall (1921). In all four experiments performed we have found that the potassium content of the plasma increases with the duration of stasis. This is in accord with the finding of Scudder (1939) and of Downman, Oliver and Young (1940) on stored blood. When the average K-contents and the M.C.F. values are plotted together against time of incubation (fig. 2)

it reveals the fact that the rise of potassium level runs almost parallel with that of erythrocytic fragility. The shift of this original impermeable kation from the cell to the plasma clearly indicates the increased permeability of cell membrane to this ion, though the permeability to other ions or molecules may not follow the same change. At any rate, the alteration of membrane permeability still remains as a possibility of primary or secondary importance in the causation of increased fragility by stasis.

The hypertonicity of the plasma may produce a double effect. In the first place, it causes shrinkage of the cell and thus delays spherocytosis. In the second place, it tends to balance up the increasing osmotic concentration of the cell during stasis, hence avoiding the outweighing inward diffusion of water and expansion of cell volume. The increase of M.C.V. and the diminution of cell volume by addition of sufficient glucose justify the above interpretation. But again, the possibility of change in membrane permeability has not been ruled out in this case.

SUMMARY

In the present investigation various experiments were designed to elucidate the mechanism underlying the increase of erythrocytic fragility induced by artificial stasis. It was found that the phenomenon of increased fragility does not occur if the cells were previously washed with buffered saline. It is also absent in the blood to which sufficient quantity of glucose or citrate has been previously added so as to make the plasma highly hypertonic. The above findings may be explained by the rapid formation and possible accumulation of osmotically active metabolites within the cell. Lactic acid is one of these substances, but probably not the only one because addition of this substance to the blood before incubation does not increase fragility to the extent anticipated if it were the sole agent. The possibility of alteration of membrane permeability by stasis which is not excluded in the present experiment is also discussed.

REFERENCES

Bergenhem, F. and R. Fahraeus. Ztschr. ges. exper. Med. **97:** 555, 1936.
Creed, E. J. Path. and Bact. **46:** 331, 1938.
Downman, C. B. B., J. O. Oliver and I. M. Young. Brit. M. J. No. 4135: 559, 1940.
Ham, T. H. and W. B. Castle. Proc. Am. Philosoph. Soc. **82:** 411, 1940.
Kramer, B. and F. F. Tisdall. J. Biol. Chem. **46:** 339, 1920.
Peters, J. P. and D. D. Van Slyke. Quantitative clinical chemistry. **1:** 610, 1931.
Scudder, J., C. R. Drew, D. R. Corcoran and D. C. Bull. J. A. M. A. **112:** 2263, 1939.
Tsai, C., J. S. Lee and C. H. Wu. Chinese J. Physiol. **15:** 165, 1940.
Waller, J. Proc. Soc. Exper. Biol. and Med. **42:** 64, 1939.
Wu, C. H. and C. Tsai. Chinese J. Physiol. **15:** 289, 1940.

CHEMICAL CHANGES IN THE RABBIT HEART DURING HYPERTROPHY[1, 2]

GEORGE H. HITCHINGS, MARGARET A. DAUS AND JOSEPH T. WEARN

From the Department of Medicine, School of Medicine, Western Reserve University, Cleveland, Ohio

Received for publication June 11, 1942

The present paper reports the results of chemical analyses of rabbit hearts made at intervals after the rupture of an aortic valve leaflet. Such hearts undergo a rapid hypertrophy. It is of interest, therefore, to compare the chemical changes in this tissue with those in other tissues undergoing rapid growth. Moreover, chemical differences between the normal and hypertrophied myocardium might be expected to result from the decreased capillary concentration (1) and consequent longer pathways of diffusion of nutrients and metabolites, of the latter.

EXPERIMENTAL METHODS. *Material.* New Zealand white male rabbits weighing about 2.2 kgm. were used for the greater number of the experiments reported.

Preparation of experimental animals. An aortic valve leaflet was ruptured by means of a sound introduced under aseptic conditions through the carotid artery (1). A sham operation, through all steps except the rupture of the valve, was carried out on an *operated control* group of animals. These hearts were taken for analysis 3 days after operation.

Preparation of tissues for analysis. Twenty milligrams of sodium pentobarbital, per kilo of body weight, were administered intravenously. Blood was drawn and defibrinated under oil. The injection of anesthetic was repeated. The heart was removed while beating, trimmed, blotted off and weighed to the nearest decigram. The left ventricle and interventricular septum were dissected out and used for analysis. The aortic valves and surrounding tissues were kept intact, and the insufficiency of the valve was confirmed for each hypertrophied heart.

Chemical methods. The methods of Hastings and co-workers (2, 3, 4) were used except as noted.

The amount of *blood* in the tissues was determined by extracting the hemoglobins with 10 volumes of 0.4 per cent ammonia water for 24 hours at 4°. The total hematin was estimated and a correction was made for myoglobin as determined by Watson's method (5).

Sodium was determined in the *potassium* chloroplatinate (6) filtrate. In order to avoid the tendency of the precipitate to float when centrifuged, and supersaturation (4), the use of alcohol to induce precipitation (7) was adopted.

[1] A report of this work was presented at the meeting of the American Society of Biological Chemists at Boston in March, 1942. Fed. Proc. **1**: 116, 1942.

[2] This work was made possible by a grant from the Commonwealth Fund.

Phosphorus fractions. Acid soluble phosphate (AP) was determined by the extraction of about 0.5 gram of tissue with 10 cc. of 0.75 N nitric acid per gram, for 2 hours. The mixture was centrifuged and the residue was extracted successively for hour periods with 10 cc. per gram of 1, a mixture containing equal volumes of 95 per cent ethanol and diethyl ether; 2, a mixture of 10 volumes of alcohol and 90 volumes of ether, and 3, finally was extracted overnight with the same volume of petroleum ether. The combined extracts comprise the *lipid phosphorus* fraction (LP), while the residue is designated the *nuclear phosphorus* (NP). From the amount of phosphorus found in the lipid extract there was subtracted a correction for the amount of acid soluble phosphorus left in contact with the residue from the acid extract.

Calculations and assumptions. The assumptions, methods of calculation and symbols of Hastings and Eichelberger (2) were followed in the description of the results of analysis.

In order to make use of the heart weight-body weight ratio as a measure of the degree of hypertrophy the analytical figures have been treated in certain calculations as if they were representative of the whole heart. Since the tissue analyzed is a rather constant fraction of the whole (8) (9) such calculations should be valid for comparative purposes.

Results. The results of the analyses of 85 rabbit hearts are summarized in table 1. Each value represents the average of analyses of the number of individual hearts shown in the second column. The first 2 lines of table 1 show the chloride, water and phase data for two control series, 8 normal and 6 operated controls. The 2 series do not differ significantly in any particular. The third line shows the combined average values of all control animals. The average ratio of heart weight to body weight, 2.22 grams per kgm., may be compared with the values 1.97, 2.16 and 2.25 reported by Herrmann and collaborators (8, 10).

The next three lines of table 1 give the results of analyses made 1, 2, or 3 days, respectively, after operation. A moderate increase in heart weight is to be noted. Chemically, the outstanding difference between these and normal hearts is to be found in the elevated water and extracellular electrolyte content of the hearts of the operated groups. This is reflected in the chloride, sodium and extracellular phase averages. For example, on the third post-operative day the average extracellular phase was found to be 280 as compared with an average normal value of 241. It is also apparent in the values for tissue solids and nitrogen.

The magnitude of the changes involved is brought out more clearly by reference to the data for the analyses of the individual hearts (table 2). The highest value for the $(F)_{Cl}$ found in any normal heart was 264. In 2 of the 4 hearts analyzed 1 day after operation, and in 1 of 3 hearts analyzed 2 days after operation the values were greater than this highest normal value. On the third post-operative day, the extracellular phase was greater than 270 grams per kgm. in 8 of the 14 hearts analyzed and, with 1 exception, the value was greater than the normal average in each of the remaining hearts.

TABLE 1

Average analyses of normal and hypertrophied rabbit hearts

Analyses are referred to 1 kilo of blood-free, fat-free tissue or phase

DESCRIPTION	NUMBER OF EXPERIMENTS	H.W./B.W.	Cl	F_{Cl}	S	$[H_2O]_C$
		grams/kgm.	*m.eq.*	*grams*	*grams*	*grams*
Control	8	2.19	27.7	245	214	720
Operated control	6	2.25	26.4	235	210	722
All controls	14	2.22	27.2	241	212	721
σ		0.13	2.2	18	4	8
P.O. 1 day	4	2.40	32.1	275	209	715
P.O. 2 days	3	2.49	28.9	257	198	737
P.O. 3 days	14	2.69	32.1	280	198	729
σ		0.34	3.9	31	10	10
P.O. 4–6 days	7	2.61	27.8	239	202	738
σ		0.28	1.2	6	5	7
P.O. 7 days	7	2.81	26.5	231	201	742
σ		0.26	2.1	16	5	9
P.O. 8–9 days	3	2.61	27.4	238	210	726
P.O. 13–16 days	6	3.25	23.3	205	204	746
σ		0.24	3.3	25	7	9
P.O. 19–24 days	5	3.62	24.6	220	211	737
σ		0.43	1.7	18	5	9
P.O. 31 days	5	3.36	23.0	207	204	746
σ		0.29	1.8	11	4	6
P.O. 64 days	3	3.11	24.7	208	212	736
P.O. 80 days	1	3.85	22.1	195	210	742
P.O. 138–147	6	3.72	27.3	232	202	741
σ		0.35	1.4	14	3	5
P.O. 152–257	7	3.13	25.9	219	201	744
σ		0.47	1.6	11	5	6

Symbols used: H.W., heart weight; B.W., body weight; F, extracellular phase; $[H_2O]_C$, water per kilo intracellular phase; AP, acid soluble phosphorus; LP, lipid phosphorus; NP, residual phosphorus; S, tissue solids; σ, standard deviation; P.O., post-operative.

TIME AFTER OPERATION	NUMBER OF EXPERIMENTS	Na	F_{Na}	K	AP	LP	NP	N*
days		*m.eq.*	*grams*	*m.eq.*	*mM*	*mM*	*mM*	*grams*
Control	6	35.4	247	82.5	41.8	27.8	11.1	28.4
		2.7	17	1.7	2.3	2.2	1.4	0.7
3	8	42.1	303	77.4	35.1	26.8	10.5	26.7
		3.6	29	2.0	2.7	1.9	1.2	1.2
7	4	35.1	247	79.4	37.4	28.4	10.6	27.2
13–15	4	30.9	224	79.2	36.7	26.5	10.8	27.5
31	4	33.4	239	80.0	39.3	26.2	10.3	28.1
64	3	31.0	215	73.7	42.2	27.7	10.5	
138–147	6	35.3	245	74.9	38.9	24.3	10.8	27.8
		2.0	18	3.1	3.1	2.5	1.2	0.9
152–257	7	37.0	257	75.1	39.0	25.2	9.3	27.6
		3.1	21	2.3	2.3	2.1	0.8	1.0

* Not corrected for blood content of the tissue.

None of the hearts analyzed on the fourth or subsequent days was found to have an extracellular phase in excess of the proportion found in the normal hearts. An interpretation of these results is found in the assumption that there exists a period of extracellular edema of considerable magnitude but of relatively short duration. Because the edema is transient, it is not always possible to demonstrate it, for the time of its appearance may not coincide with the time chosen for analysis.

While the major part of the increased heart weight on the third post-operative day can be attributed to an extracellular edema, there is also demonstrable at that time an increase in the intracellular phase. This is brought out in figure 1 in which the weight of intracellular phase per unit of body weight: $\frac{\text{H.W.}}{\text{B.W.}}\left(1 - \frac{F_{Cl}}{1000}\right)$ has been plotted for each period studied. Cell growth appears

TABLE 2

Analyses of hearts three days after valvulotomy

Analyses are referred to 1 kilo of fat-free, blood-free tissue

H.W./B.W.	F_{Cl}	S	$\{H_2O\}_C$	F_{Na}	K	AP	N*
grams/kgm.	*grams*	*grams*	*grams*	*grams*	*m.eq.*	*mM*	*grams*
2.92	268	210	716	281	75.9	42.0	27.5
3.01	250	194	745	278	79.8	35.2	27.8
3.03	306	185	741	310	74.9	32.7	25.1
3.00	287	212	707	285	76.5	34.4	27.2
3.06	321	183	735	347	74.6	34.3	24.8
2.43	262	194	740	284	79.4	34.8	26.5
2.47	275	202	726	288	78.9	32.7	26.4
2.43	250	204	731	250	79.2	34.6	28.0
2.46	260	209	722				
2.68	309	192	727				
3.32	354	178	729				
2.28	231	202	741				
2.33	275	206	719				
2.22	273	201	727				

* Not corrected for blood content of the tissue.

to proceed in a rather regular fashion for about 3 weeks. Further increase during longer intervals could not be clearly demonstrated.

As the growth of the intracellular phase continues there is a tendency for the proportion of this phase in the tissue to reach a value somewhat greater than normal. This is brought out in the extracellular phase data for the 13 to 16 day, and subsequent, periods. Thus the average $(F)_{Cl}$ for all hearts analyzed from 3 to 80 days after valvulotomy is 209, σ 12, compared with the normal value of 241, σ 18. The elevated proportion of intracellular phase tends to magnify the tissue content of all intracellular substance. This tendency is counterbalanced, however, by the higher water content of the intracellular phase ($\{H_2O\}_C$) of all the hypertrophied hearts.

The chemical changes in the intracellular constituents which occur during hypertrophy are made apparent by calculating the composition of the "increment of growth" for each period studied, by means of an equation similar to that of Yannet and Darrow (28) $K\Delta = \frac{C_H K_H - C_N H_N}{C_H - C_N}$, where C represents the intracellular phase in grams per kilogram of body weight; K the composition of the intracellular phase with respect to a given constituent, and the subscripts Δ, H, and N refer to the increment of growth, the hypertrophied heart and the normal heart, respectively. The results of the calculations are presented in table 3.

For each series of experiments the composition of the intracellular phase is given in the first line and the composition of the increment of growth on the second. The data of the third line represent the composition of the increment of

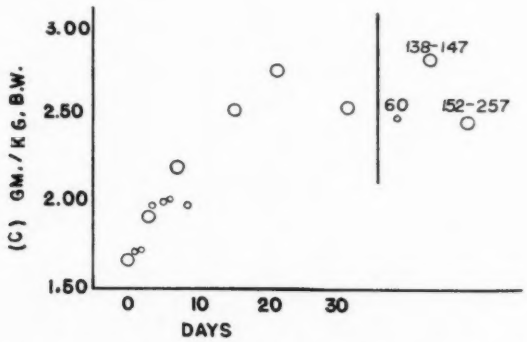


Fig. 1. Increase of intracellular phase. Large circles represent averages of 5 or more analyses; small circles 2 or 3 analyses. The ordinates represent the intracellular phase in grams per kilo of body weight.

growth referred to a normal intracellular solid content, i.e., $K\Delta$ corr. $= K\Delta \frac{(1000 - \{H_2O\}_C)_N}{(1000 - \{H_2O\}_C)\Delta}$.

When the concentrations of cellular constituents are compared on the basis of the same quantity of cell solids, it is seen that in general the concentrations are similar to those of normal tissue. However, the acid soluble phosphorus concentration appears to be less than normal during the early periods, but to reach a normal level after some weeks and to be maintained at this level throughout the period studied. In contrast, potassium and phospholipin phosphorus are present in less than normal concentrations at the longer periods.

The difference in intracellular water concentration between hypertrophied and normal hearts is much too large to be accounted for on the basis of a possible difference in connective tissue content (see e.g., (3) (11)). Moreover, no significant difference in collagen nitrogen was found. The average collagen nitrogen (12) of 5 normal hearts was found to be 5.88 grams of nitrogen per kilo, σ 0.78, compared with 5.09, σ 0.91, for 16 hypertrophied hearts.

An occasional experimental animal died of what appeared to be congestive heart failure with anasarca. It was never found possible to bring such a specimen to analysis *ante mortem*, but the analysis, *post mortem*, of 1 specimen is shown in table 4 (expt. 100). This animal was found dead 82 days after operation and was stated to have been alive 17 hours earlier. Analysis revealed a very high water content, sodium and chloride concentrations greater than twice normal, and a potassium concentration about 70 per cent of normal.

TABLE 3

Composition of increments of growth of intracellular phase

Analytical values are referred to 1 kilo of intracellular phase

	TIME AFTER OPERATION	INCREMENT	H_2O	K	AP	LP
	days	*grams/kgm. B.W.*	*grams*	*m.eq.*	*mM*	*mM*
	Normal	0.25	721	108	54.6	36.3
A	3	0.25	729	114	49.6	37.2
B			780	134	21.2	43.2
C				170	26.9	54.8
A	13–15	0.90	746	102	46.2	33.4
B			792	85	32.3	28.0
C				114	43.3	37.6
A	31	0.92	746	101	49.5	33.0
B			792	83	42.0	27.0
C				111	56.3	36.2
A	138–147	1.16	741	99	50.7	31.6
B			776	82	46.5	24.8
C				99	55.9	29.8
A	152–257	0.76	744	96	50.0	32.3
B			794	64	42.0	23.4
C				86	56.8	31.7

A—Composition of intracellular phase of heart.
B—Composition of increment of growth of intracellular phase.
C—Composition of increment corrected to normal solid content.

The results of experiments designed to determine the chemical changes which occur in the myocardium *post mortem* are given in the final 6 columns of table 4. In each instance 50 mgm. per kilo of sodium pentobarbital were injected intravenously. Except as indicated (expt. 141) respirations ceased in about 5 minutes, and the heart failed about 5 minutes later.

The water content of the myocardium rises rapidly during failure under anoxic conditions (expts. 140, 141). The sodium and chloride content rise somewhat, but much greater increases in extracellular electrolytes appear to occur when the heart is allowed to remain *in situ* after death. Similarly, losses in potassium

and phosphorus appear to occur only during *post mortem* autolysis. These findings suggest that the loss of intracellular electrolytes occurs not only as a result of exchanges across cell membranes, but also as a result of a mechanical expression of fluid from the tissues as *rigor mortis* sets in.

DISCUSSION. The data presented here show that within a short period after aortic regurgitation is produced experimentally, the myocardium takes up considerable amounts of sodium chloride and water. An increase in the extracellular phase of heart muscle amounting to 10 per cent was found by Hastings et al. (13) to follow a short ligation of a coronary artery. This was believed to be the result both of an increase in interstitial fluid and changes in permeability due to injury. It appears that the increase in extracellular phase observed in the early stages of aortic insufficiency reflect chiefly an increase in interstitial fluid, rather

TABLE 4

Post mortem changes

Analytical values are referred to 200 grams of blood-free, fat-free solid

	NORMAL	EXPERIMENT						
		100	141†	140	137	119	133	136
H.W./B.W., grams/kgm	2.25	4.33	1.91	2.48	2.25	2.46	3.09	2.23
Temperature, post mortem	c.18°				4°	4°	23°	23°
Time after death, hours	8	17	0	0	17	17	17	17
H_2O, grams	752	917	815	943	899	899	887	899
N*, grams	27.1	24.0	27.8	27.5	26.9	26.4	26.9	
Cl, m.eq	25.1	56.5	28.3	31.2	36.7	32.9	47.9	47.1
Na, m.eq	33.7	72.8	35.6	40.7	53.3	40.4	62.4	73.2
K, m.eq	78.0	55.8	77.8	81.6	66.6	65.3	63.0	68.2
AP, mM	39.8		39.9	38.6	40.4	34.3	32.9	37.1
LP, mM	26.5		25.2	24.9	26.7	28.3	26.0	28.3
NP, mM	11.3		11.0	10.9	8.1	11.5	9.7	9.1

* Nitrogen values were not corrected for blood content of tissue.

† The heart stopped beating 3 minutes after anaesthetic was given and was removed for analysis immediately.

than injury, for the intracellular elements undergo a rapid growth, and concentrate potassium at least as fast as is required by the synthesis of new intracellular material. The transient increase in extracellular phase and the other chemical changes in early hypertrophy are much the same as the changes which occur in other tissues when stimulated to rapid growth by means of hormones (14, 15). This suggests that such a pattern of changes may accompany the rapid growth of tissues whatever the stimulus for growth may be.

Chemically the most striking differences between the hypertrophied and the normal hearts during the intermediate periods (1 to 3 mos. postoperative) are the higher intracellular phase and intracellular water of the hypertrophied tissue. The increased intracellular phase of moderately hypertrophied hearts gives rise at times to apparent supernormal concentrations of intracellular constituents

when these are expressed per unit of tissue or, especially, of tissue solids. This tendency is apparent with respect to potassium in the analyses of many of the individual specimens of the present study, but in the averages it is obscured by the presence in each group of individuals showing the potassium loss characteristic of hypertrophy of longer duration. An increased proportion of intracellular phase presumably accounts for the rise in creatine concentration reported to occur in slight hypertrophy (16).

The composition of the hypertrophied heart 4 months or longer after operation indicates that there is a tendency for the heart to lose intracellular constituents, in these studies chiefly potassium and phospholipin. The intracellular water content remains elevated which presumably indicates the persistence of some metabolic abnormality, whether a change in hydrogen ion activity, or in the concentration of metabolically active substances in one or both phases. Concentrations of creatine, and of acid-soluble, but not phospholipin, phosphorus lower than normal have been reported to occur in the rabbit heart after several months of aortic insufficiency (16) (17). Myocardial insufficiency in the human heart is accompanied by loss of creatine, potassium and phosphorus and by increases in extracellular electrolytes and water (17) (18) (19) (20). To some extent the latter changes might be the result of ionic and water exchanges during the final stages of failure and *post mortem* if such changes in the human heart follow the pattern found in the rabbit heart.

SUMMARY

When aortic insufficiency is produced in the rabbit heart by rupturing an aortic valve leaflet, rapid changes occur in the chemical composition of the myocardium. During the first 3 days there is a transient increase of extracellular phase of considerable magnitude. The intracellular phase appears to hypertrophy at a more or less constant rate for several weeks after which further increases cannot be clearly distinguished. The hypertrophied hearts at intermediate periods are characterized by a proportion of intracellular phase somewhat greater than normal but of approximately normal composition except for an increased intracellular water content. At later periods a tendency for a loss of intracellular constituents is observed.

REFERENCES

(1) Shipley, R. A., L. J. Shipley and J. T. Wearn. J. Exper. Med. **65:** 29, 1937.
(2) Hastings, A. B. and L. Eichelberger. J. Biol. Chem. **117:** 73, 1937.
(3) Manery, J. F., I. S. Danielson and A. B. Hastings. J. Biol. Chem. **124:** 359, 1938.
(4) Manery, J. F. and A. B. Hastings. J. Biol. Chem. **127:** 657, 1939.
(5) Watson, R. H. Biochem. J. **29:** 2114, 1935.
(6) Shohl, A. T. and H. B. Bennett. J. Biol. Chem. **78:** 643, 1928.
(7) Salit, P. W. J. Biol. Chem. **96:** 659, 1932.
(8) Herrmann, G., G. Decherd, P. Erhard and E. M. Schwab. Proc. Soc. Exper. Biol. and Med. **33:** 409, 1935–36.
(9) Schwab, E. H., G. Herrmann and J. F. Connally, Jr. Proc. Soc. Exper. Biol. and Med. **33:** 410, 1935–36.
(10) Herrmann, G. and G. M. Decherd, Jr. Ann. Int. Med. **13:** 794, 1939.

(11) Muntwyler, E., R. C. Mellors, F. R. Mautz and G. H. Mangun. J. Biol. Chem. **134:** 367, 1940.
(12) Spencer, H. C., S. Morgulis and V. M. Wilder. J. Biol. Chem. **120:** 257, 1937.
(13) Hastings, A. B., H. L. Blumgart, O. H. Lowry and D. R. Gilligan. Tr. A. Am. Physicians **54:** 237, 1939.
(14) Logan, M. A., J. E. VanderLaan and W. P. VanderLaan. J. Biol. Chem. **133:** lxii, 1940.
(15) Talbot, M. B., O. H. Lowry and E. B. Astwood. J. Biol. Chem. **132:** 1, 1940.
(16) Herrmann, G., G. Decherd, E. H. Schwab and P. Erhard. Proc. Soc. Exper. Biol and Med. **33:** 522, 1936.
(17) Herrmann, G. and G. M. Decherd. Ann. Int. Med. **12:** 1233, 1939.
(18) Myers, V. C. and G. H. Mangun. J. Lab. Clin. Med. **26:** 299, 1940.
(19) Mangun, G. H., H. S. Reichle and V. C. Myers. Arch. Int. Med. **67:** 320, 1941.
(20) Calhoun, J. A., G. E. Cullen, G. Clarke and T. R. Harrison. J. Clin. Investigation **9:** 393, 1930–31.

REFLEXES FROM THE LIMBS AS A FACTOR IN THE HYPERPNEA OF MUSCULAR EXERCISE[1]

J. H. COMROE, JR. AND CARL F. SCHMIDT

From the Laboratory of Pharmacology, University of Pennsylvania, Philadelphia

Received for publication October 10, 1942

It has been known for many years that in the two commonest and most important physiological emergencies calling for increased pulmonary ventilation, viz., muscular exercise and anoxemia, the hyperpnea is not referable to an increase in the amount of any known chemical stimulant in the arterial blood. Various suggestions have been advanced to explain this discrepancy, such as the existence of an unknown chemical excitant (3, 9), inadequacy of existing chemical methods to detect the responsible acid change in the arterial blood (5), or an acid shift within the respiratory center though not in the arterial blood (4, 24), but the view most widely held until recently was that both hyperpneas are brought about by an increase in the sensitivity of the respiratory center to its normal chemical stimulus (12, 16). The brilliant studies of Heymans and his collaborators (10, 11), amply confirmed by others (see 19), have shown that this increased sensitivity during anoxemia actually is brought about by excitatory nerve impulses from the carotid and aortic chemoreceptors, which furnish an adequate explanation for the coexistence of hyperpnea, hypocapnia, and alkalosis during anoxemia. In view of this course of events with regard to one of these physiological emergencies it is reasonable to suppose that excitatory reflexes may also be prominently involved in the other, but so far little attention seems to have been given to this possibility. Yet Harrison and his collaborators (6, 7) concluded from experiments on men and dogs that reflexes aroused by movements of the limbs play a part in the hyperpnea of exercise, and Alam and Smirk (1) presented evidence that a chemosensitive reflex system capable of stimulating the vasomotor center is present in the extremities of man. In view of their potential importance it is rather surprising that these experiments have not been repeated. Since Harrison's results were not very striking and since Alam and Smirk present no data bearing on the respiration, we undertook to repeat both sets of experiments from the standpoint of a possible explanation for the hyperpnea of muscular exercise.

1. *Experiments of the Alam and Smirk Type on Man.* These were performed on healthy male subjects (staff members, medical students, and technicians) reclining on a couch. A comfortable rubber face mask fitted with valves was used and the expired air was passed through a gas meter for measurement. A pneumogram was also made. Blood pressure was measured at frequent intervals in the left arm by the Riva-Rocci method and another

[1] This investigation was partly financed through the National Committee for Mental Hygiene from funds granted by the Committee on Research in Dementia Praecox founded by the Supreme Council, 33rd Scottish Rite, Northern Masonic Jurisdiction, U. S. A.

cuff was applied to the right arm so that the arterial inflow could be cut off when desired. Exercise of the right forearm and hand was brought about by flexion of the fingers in time with a metronome beating once per second, each effort raising a weight of 1360 grams a distance 5 to 7 cm. With certain exceptions which will be indicated, a complete experiment comprised five periods, each lasting two minutes: *1*, resting control (two minutes of observation after a steady state had been shown to be present); *2*, occlusion of the circulation

TABLE 1

SUBJECT	PER CENT CHANGE IN																SENSATIONS			
	Resp. rate				Resp. min. vol.				Systolic B-P				Diastolic B-P							
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
P. D.	−14	−3	0	+24	0	−12	+23	+35	0	−2	+5	+9	0	0	+14	+16	0	0	0	T
	−18	0	0	0	0	0	0	0	0	0	+8	+6	0	0	+6	+6	0	0	0	0
	−10	0	−12	−16	+12	0	+16	+16	0	0	+6	+14	0	0	+2	+2	0	0	0	0
J. C.	+5	0	+14		+50	−8	+61		+2	0	+16		+15	0	+6		0	NT	WN	
	+8	0	+18	+9	+15	+5	+16	+23	0	0	+8	+11	+3	0	+7	+15	0	0	P	PN
	+8	0	+40	+20	+13	0	+30	+20	+5	+2	+5	+9	+8	+2	+6	+19	0	0	WP	
	+25	0	+30	+10	+30	0	+45	+20	0	0	+2	0	0	0	+9	+16	0	0	W	NT
	+41	0	+25	+20	+37	0	+40	+61	0	0	+2	+13	+5	0	+15	+12	0	0	ND	N
	+31	−4	+7	0	+25	−13	+28	+12	+2	0	+3	+9	+3	0	+4	+6	0	0	W	DT
J. R.	+15	0	−27	0	0	−14	−16	0	0	0	+4	+6	+5	0	+9	+12	0	N	WP	N
	−12	−12	−11	+11	−10	0	+15	+25	0	0	+4	+6	0	0	+17	+17	0	0	NP	PP
	0	0	0	0	0	0	+75	+30	0	0	+10	+4	0	0	+6	0	0	0	0	0
R. E.	+40	+4	+9	−9	+40	+14	+32	0	0	0			0	0			0	0	NP	P
	0	0	0	0	+36	+12	+84	+26	0	0	+4	+6	0	0	0	+5	0	0	P	NPT
O. H.	0	0	+20	+20	0	+25	−22	+12	0	0	+4	0	0	0	+3	0	0	D	0	PT
	+47	+23	+62	+10	+30	0	+29	−3	0	0	+2	+2	0	0	+5	+5	0	0	0	0
C. S.		−11	+55	+22	0	0	+33	+7	0	0	+3	+5	0	0	+5	+5	0	NT	W	NT
J. W.	−11	0	0	−12	−8	0	+13	+3	+7	0	+10	+12	+15	0	+19	+19	0	T	T	P
H. P.	+11	−20	+16	−5	+10	+12	+31	0	+13	+2	+10	0	+12	+7		0	0	T	NP	NP
J. S.	+33	0	+33	0	+20	0	+28	0	+9	0	+6	+6	+2	0	+21	+21	0	T	WT	T
F. H.	+8	0	+15	0	+11	0	+7	+3	0	0	+2	+4	0	0	+3	+3	0	0	0	0
I. S.	+4	0	+9	+13	+27	+2	+35	−4	0	0	0	+5	0	0	0	+4	0	0	P	P
Average..	+10	−1	+14	+6	+14	+0.4	+28	+14	+2	0	+6	+6	+3	0	+7	+9				

1 = change from control to exercise alone.
2 = change from control to ischemia alone.
3 = change from control to exercise plus ischemia.
4 = change from control to continued occlusion after exercise.
Sensations: D, discomfort; N, numbness; P, pain; T, tingling; W, weakness; 0, none; blank, not recorded.

in the right arm by inflation of the cuff by a pressure higher than the systolic arterial level; release and recovery to normal, then *3*, exercise of the right forearm and hand with circulation intact; rest and recovery, then *4*, repetition of the exercise with the circulation cut off (terminated as soon as any unpleasant sensations were experienced, often in less than two minutes); *5*, cessation of the exercise without restoration of the circulation. This report is based on the results of 23 satisfactory experiments of this type on 11 subjects. The results are summarized in table 1.

The *respiratory* findings at the various stages of these experiments justify brief comments. First, since simple ischemia did not cause hyperpnea, it follows that there are in the forearm and hand of man no chemoreceptors comparable in sensitivity to those in the carotid bodies, which are strongly stimulated by total ischemia of considerably less than two minutes' duration (23). Second, voluntary exercise, even of a relatively small mass of muscle, caused a distinct (average 14 per cent) increase in respiratory minute volume even with intact circulation. Third, exercise in the presence of ischemia brought about a much greater (average 28 per cent) increase in pulmonary ventilation, which might have been due either to a specific reflex aroused by chemical substances acting locally in the muscles (since their escape was prevented by the inflated bandage), or to a nonspecific reflex of the pain type. Our findings point toward the latter explanation, because in 15 of the 22 experiments respiration returned toward normal when the exercise was discontinued even though the ischemia persisted and the concentration of chemical stimuli could not have diminished. That pain was the dominant factor here was further suggested by the variability of the response of a given individual at different times (which is difficult to reconcile with the requirements of a specific reflex system subserving an important function), and by the fact that, in all of the five instances in which the hyperpnea was greater during the final period of ischemia after exercise, pain also persisted; all of the other subjects felt more comfortable as soon as they ceased exercising.

Our findings with regard to the blood pressure seem to us to be without significance. They are much less striking than those reported by Alam and Smirk (1). Whether this is because our subjects were not exposed to as severe pain as theirs, or because we did not happen to encounter any of the unusually reactive individuals who comprised only one-fourth of those tested by Alam and Smirk, we are not prepared to say.

In order to exclude the influence of pain and other psychic factors we next undertook a series of experiments on anesthetized animals in which observations could be made along the same general lines but under more controllable conditions.

2. *Experiments of the Alam and Smirk Type on Animals.* Our purpose was to test the effects of exercise alone, of ischemia alone, and of exercise plus ischemia. To induce "exercise" without direct sensory involvement we resorted to stimulation of ventral spinal roots.

A. *Cats.* The animals were narcotized by chloralose (0.05 gram per kgm. intravenously) or barbital sodium (0.35 gram per kilo intraperitoneally). The ventral lumbar spinal roots were exposed by Sherrington's method (20), cut free on the central side, and placed on insulated electrodes raised above the cord to obviate escape of current. The stimulus was obtained from a thyratron stimulator, the frequency being 4 per second throughout and the strength adjusted so as to elicit maximal muscular activity. In some cases only the 7th lumbar pair of ventral roots were stimulated, in others the 6th lumbar, and in a few the first sacral also, were added. Respiration was recorded by a small oxygen-filled spirometer to which the animal's tracheal cannula was connected through a canister containing soda-lime. Blood pressure was registered from a carotid artery by means of a mercury manometer, 25 per cent $Na_2S_2O_3$ serving as the anticoagulant.

We made 29 observations of the effects of "muscular exercise" thus induced in 15 cats, and in every case there was some increase in respiratory minute volume. Other noteworthy results were the following: First, the stimulation involved slight to moderate increase in *depth* of breathing, rate being increased seldom and never markedly; second, there was a latent period of at least 15 seconds between the start of the stimulation and the onset of the increased depth of breathing; third, the depth increased progressively to reach a plateau; and finally, recovery was gradual and slow after the stimulus was withdrawn. Ischemia alone (produced by clamping the femoral arteries and veins or the abdominal aorta and vena cava) produced no changes in respiration. "Exercise", tested 14 times in the presence of ischemia, regularly had less effect on breathing than it had when the circulation was intact, and often it had no effect at all. When the vessels were reopened, however, hyperpnea promptly appeared. Blood pressure commonly fell during the "exercise"; it never rose even when the vessels were closed during the "exercise" period.

Characteristic examples of these findings are shown in figure 1. The results strongly suggest that the hyperpnea of "muscular exercise", induced in the cat by stimulation of ventral spinal roots, is largely due to liberation into the blood stream of chemical products which act either on the center or on chemosensitive nerve endings elsewhere than in the leg. This was confirmed by the effect of transection of the spinal cord in the lower dorsal region in 5 cats. After that operation "exercise" still increased breathing much as it had done before. If there is any peripheral reflex component in the hyperpnea of this type of "exercise" in the cat, it must therefore be small.

These results with cats are quite unlike those obtained in man, in whom the hyperpnea of voluntary exercise was definitely increased by ischemia and therefore could not have been due to direct stimulation of the center by chemical substances. This suggested that species differences may exist.

B. *Dogs.* These were prepared in the same way as the cats. The anesthetic was sodium barbital (0.25 gram per kilo intravenously) or chloralose (0.03 gram per kilo intravenously after 2 mgm. of morphine per kilo subcutaneously).

Out of 39 tests of ventral root stimulation in 11 dogs, 35 showed definite respiratory stimulation, the increase in minute volume varying from 8 to 200 per cent and averaging 62 per cent. In one there was no change (only one root was stimulated here) and in 3 there was pure depression of breathing, for which we have no explanation. The noteworthy features were as follows: First, the stimulant effect was mainly on the *rate* of breathing; depth might be unchanged, slightly increased, or slightly decreased. Second, the polypnea came on immediately with the start of the stimulation and was maximal within the first 30 seconds, after which it often diminished during the remainder of the period of stimulation. Third, when the stimulus was withdrawn breathing returned to normal almost immediately. Fourth, the response was unaltered when the femoral vessels were occluded during the stimulation, and there was no consistent effect on breathing when the vessels were reopened after the stimulation. Finally, transection of the spinal cord in the lower dorsal region completely abolished this polypnea

in each of 4 dogs on which the point was tested. Section of the dorsal lumbar roots likewise abolished the effect in other animals.

Typical examples of the dog experiments are shown in figure 2.

That these findings were not due to escape of current to the dorsal roots or to the cord itself was shown by the following: First, the polypnea did not increase further when the strength of the stimulus was increased above the level that sufficed to produce maximal muscular activity. Second, elevation of the electrodes to the fullest extent permitted by the cut ventral roots had no influence on the results. Third, the response was entirely

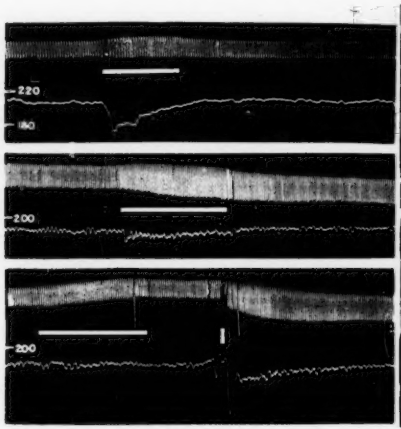


Fig. 1

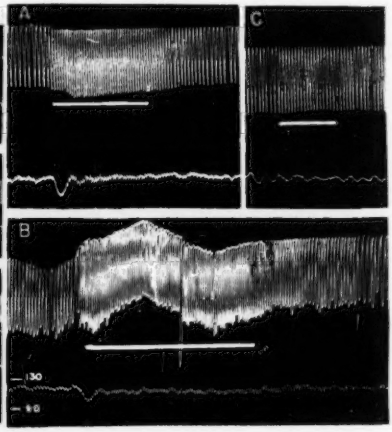


Fig. 2

Fig. 1. Cat. Chloralose anesthesia. In this and the following kymographic tracings upper record is spirometric tracing of respiration and lower record represents carotid blood pressure. Horizontal white bar indicates period of electrical stimulation of cut peripheral ends of L 7 anterior spinal roots. Numbers at left show actual level of blood pressure. Upper tracing—intact cat. Middle tracing—cord has been sectioned at level of emergence of L 4 roots. Lower tracing—cord cut, abdominal aorta and inferior vena cava clamped at start of record—released at vertical white bar.

Fig. 2. Dog. Morphine and chloralose anesthesia. Arrangement as in figure 1. L 6 and 7 anterior spinal roots stimulated. A, Intact dog. B, Abdominal aorta and inferior vena cava clamped at start of record (irregularities in spirometric tracing are due to manipulations of inflow of oxygen into the system). C, Cord cut at level of emergence of L 4 roots (vessels open).

lacking when the stimulation was repeated after the corresponding spinal nerves had been cut outside the vertebral canal; under these circumstances movements of the legs were also lacking, but escape of current into the dorsal roots or cord should not have been prevented.

We conclude that the respiratory stimulation associated with "exercise" induced by ventral root stimulation in the dog, unlike the corresponding effect in the cat, is essentially due to afferent nerve impulses from the limbs. Our next step was to determine whether these impulses arose from proprioceptors

responding to changes in length or position, or from chemoreceptors responding to changes in muscle metabolism. The latter seemed unlikely because the response was not appreciably enhanced by ischemia during the stimulation, but the possibility remained that adaptation (or actual deterioration in reactivity) might occur when the ischemia was as nearly absolute as it was in these experiments.

3. *Attempts to Demonstrate Chemoreceptors in the Limbs.* These experiments were of three general types: *a*, crossed perfusions in which blood from a donor animal was circulated through one or both hind limbs of the recipient by means of a perfusion pump; *b*, auto-transfusion experiments involving injection into one femoral artery of blood collected from the opposite femoral vein during "exercise", ischemia, and "exercise" plus ischemia on the side of collection; *c*, intraarterial and intramuscular injection of various chemical substances known to be associated with muscle metabolism, as well as some others. Cats and dogs were used for all three sets. It may be said at once that no definite evidence of the presence of a specific, physiologically important chemoreflex system has been obtained by any of these procedures. Detailed descriptions of the methods employed and of the individual results are therefore unnecessary, but a brief account seems desirable.

The crossed perfusion experiments were intended to test the ability of blood rendered anoxic, hypercapnic, or acidic (by appropriate manipulation of the donor) to cause a reflex hyperpnea in the recipient. No signs of any such effect were seen.

In the auto-transfusion experiments blood was collected from a branch of the femoral vein into a syringe, heparin was added to prevent clotting, and the same blood (5 to 20 cc.) was then injected into the opposite femoral artery through a suitable cannula tied into the profunda femoris, the main artery being clamped above this point during the injection. Even when the collection was made during stimulation of the muscles with the artery closed (it was opened for brief periods to permit the collection of blood from the vein), and although the appearance of the blood indicated almost complete change to reduced hemoglobin (which may be taken as an indication of the amounts of metabolic products that must have been present), there was no effect on breathing when the blood was injected into the opposite femoral artery. The same animals were responsive to intra-arterial injections of KCl (see below), so that their leg reflex systems were not unreactive.

The experiments in which various chemicals were injected intra-arterially were carried out by the same general methods as those just described. The only essential difference was the use of decerebrated cats and dogs instead of anesthetized animals because the former proved more reactive. The results were confirmatory in the main of those of the similar experiments reported by Moore and his associates (15). *Acids* (hydrochloric, lactic, phosphoric) were active when injected in concentration of 0.1 N or stronger but thrombosis of the artery frequently followed repeated injections. To determine the sensitivity to hydrogen ions we turned to buffer mixtures of sodium phosphates. They were made isotonic with NaCl and injected at 38°C. in 1 cc. dosage with the arterial inflow cut off during the injection, restored just afterward. The threshold value turned out to be about pH 6.7, which was effective only weakly and occasionally in cats. To obtain consistent and fairly strong stimulant effects a mixture of pH 6.3 or less had to be used in dogs and cats. *Potassium chloride* was the most consistently effective and repeatable of all the chemical stimulants used by us. The minimum effective dose on intra-arterial injection in cats was 0.1 mgm. in 1 cc. of warm 0.9 per cent NaCl (the latter being entirely ineffective alone), corresponding with 0.0013 M solution of KCl. This was active only weakly and occasionally; for consistent and fairly powerful effects doses of 1 to 5 mgm. in 1 cc. (0.013 to 0.065 M) were required in cats, 1 to 10 mgm. (0.13 M) in dogs). We were also able to test a number of intermediary products of muscle metabolism that were placed at our disposal by Professor Meyerhof. Positive results were obtained with a number of them but all of the active solutions proved either to contain barium (which Moore *et al.* (15) found to be highly

effective) or to be more acid than pH 6.0. We did however make some valid tests with creatine and phosphocreatine, both of which proved entirely ineffective. *Creatine* was used in 3 per cent solution in isotonic sodium phosphate buffers at pH 7.0, 6.7, and 6.5 and the amounts injected ranged from 1 to 10 cc. in decerebrated dogs and cats. *Phosphocreatine*, carefully freed of barium, was used in 0.5 per cent solution in phosphate buffers at pH 7.0 and the amounts injected (in dogs and cats) were 1 to 5 cc.

In addition to these substances, *NaCN* and *lobeline* were injected into the femoral artery and found effective only after a latent period of about 30 seconds. The inference—that the response, when it occurred, was due to an action on the carotid and aortic bodies—was confirmed by the effects after denervation of these structures. After the denervation there was no hyerpnea from doses which had previously caused a strong delayed response. *Ether* dissolved in saline was also tried and found to cause distinct hyperpnea, even in concentrations as low as 0.1 per cent. The effect came on immediately and was abolished by denervation of the limb. The implications with respect to the cause of the well-known hyperpnea of ether anesthesia (8, 17, 18) are obvious and are to be the object of further study.

Since the hydrion concentration and potassium content of muscle are known to rise during vigorous contraction, a specific chemoreflex system responding to those changes became a distinct possibility. However, when we injected these and other substances intramuscularly we found (like Moore and his collaborators (15)) that such injections were without effect. We injected warm isotonic solutions of phosphate and bicarbonate buffer mixtures, KCl, and phosphocreatine, in amounts ranging from 1 to 20 cc. and at pH ranging from 7.0 to 6.3, into the adductor, quadriceps, hamstring, gluteal, and gastrocnemius muscles of decerebrated dogs and cats, and in no case were there any definite stimulant effects, even from solutions that had strong effects when given intra-arterially.

It is difficult to conceive of a physiologically important chemo-reflex system, responsive to products of muscle metabolism, that would not be activated by any of these procedures except intra-arterial injections of foreign chemicals. We conclude that although the latter experiment shows a chemoreflex system to be present, the entire effect is probably due to pain impulses set up in or near the arterial wall and therefore has no great physiological importance. In this we confirm the findings and interpretations of Moore *et al.* (15). The increased breathing associated with ventral root stimulation, which we have shown above to be reflex in origin in the dog, therefore cannot arise from chemoreceptors. The experiments next to be described were undertaken to determine whether it is referable to proprioceptor reflexes of the type described by Harrison (6).

4. *Experiments of the Harrison Type.* These were performed on dogs and cats prepared as described above (p. 539); in addition the femora were disarticulated or transected and their proximal ends were clamped rigidly so that movements of the legs were not communicated to the trunk. The feet were tied to a rod that was moved vertically (in a few cases horizontally) by an electric motor; in all of the experiments now under discussion the rate of movement was 200 per minute and the type was intended to simulate normal running movements. Nineteen experiments of this type were performed on dogs, 12 on cats.

Dogs. A typical example is shown in figure 3. Increase in respiratory minute volume was seen at least once in each experiment during passive movements of the legs; the increase varied from 22 to 125 per cent and averaged 52 per cent. Thus we are able to confirm Harrison's (6) findings with regard to passive movements of the limbs. The respiratory effect of these movements had the same characteristics as that of active contractions induced by ventral root stimula-

tion, i.e., it was predominantly an effect on the *rate*, and the onset and recovery were both abrupt. The reflex nature of the response was proved by demonstrating both its continued presence during occlusion of the femoral blood vessels and its complete absence after section of the spinal cord or of the nerves to the hindlegs. The reflex evidently is not set up by stretching muscles or tendons because in 4 experiments traction was exerted on one or more of the tendons of the quadriceps and hamstring groups, and in no case was there any respiratory stimulation; in all these, shaking the legs still caused a typical response after all the tendons were cut through. That the knee joint is prominently concerned was suggested in 6 experiments in which continuous flexion of one knee caused distinct respiratory stimulation, and the result was unaffected

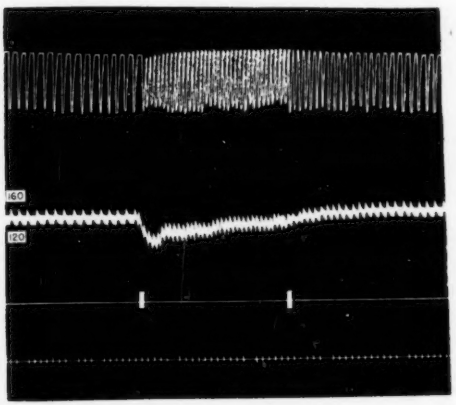


Fig. 3

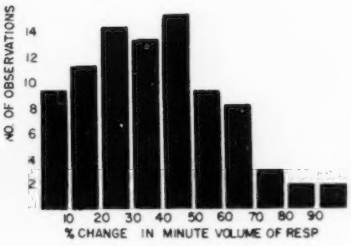


Fig. 4

Fig. 3. Dog. Anesthesia by barbital. Between signals, passive movements (200 per min.) of both hind limbs. Vessels and nerves intact.

Fig. 4. Frequency-distribution chart of respiratory response of healthy adults to passive movements (100 per min.) of left leg below knee. Each block indicates number of observations out of total of 86.

by division of all the quadriceps and hamstring tendons. Final proof of that fact was obtained by injection of 2 per cent procaine into and around the knee joint, for following this, shaking or flexion of the leg was entirely ineffective until, after about 30 minutes, the effects of the drug wore away and the response returned. In one experiment denervation of the knee was accomplished by division of all nerves in the vicinity, and this also abolished the effects of shaking or flexion. These experiments therefore show clearly that the respiratory stimulation produced in dogs by passive movements of the hindlegs is due to a reflex arising in and around the joints, particularly the knee.

Cats. The responses were both less consistent and less marked than those seen in similar experiments on dogs. As with the ventral root stimulations, the stimulant effect was mainly on the depth of breathing, it began after a latent

period, and wore away gradually after the stimulus (shaking or flexion in this case) was discontinued. Nevertheless this effect was proved to be entirely reflex by its persistence during closure of the femoral artery and vein (3 experiments) and by its absence following section of the nerves to the legs (2 experiments) or injection of 2 per cent procaine into the knee joints (2 experiments).

In view of these marked species differences, a corresponding study was carried out on man.

Healthy adult males (staff members, technicians and medical students) were used as subjects. They reclined on a padded table from which a part had been cut away so that the left leg hung free from a level about 6 inches above the knee. The left foot was tied to a stirrup on the end of a rod that was moved back and forth a distance of about 2 feet by means of an electric motor; the number of movements was 100 per minute. The subject's expired air was collected through a comfortable valved rubber face mask and was measured directly by a gas meter; a pneumogram was also recorded. The control period was usually 15 minutes (longer if a steady state had not been reached), the period of passive movement 1 to 2 minutes, and the recovery period about 6 minutes. A total of 86 tests of this sort were made in 50 subjects. The results are summarized in figure 4.

It is quite evident that the respiration of these subjects was stimulated consistently and powerfully by passive movements of one leg. Only in 4 was the increase in respiratory minute volume less than 10 per cent. In 38 it was between 20 and 60 per cent and the largest number (14 subjects) were in the group showing an increase of 40 to 50 per cent. The responses partook of the characteristics of both dogs and cats in that the increased breathing involved rate (as in the dog) and depth (as in the cat), began almost immediately (as in the dog) but often faded away gradually (as in the cat).

While the psychic factor cannot be altogether excluded in experiments such as these, the passive movements were not associated with any discomfort or pain. Direct evidence that these stimulant effects were due at least in part to afferent impulses from the leg was obtained in 4 patients in whom the experiment was carried out just before and again after the induction of spinal anesthesia. The results are summarized in table 2. The uniformity with which the respiratory response was reduced by spinal anesthesia seems to us to indicate that psychic factors were not entirely responsible. We therefore conclude that in man, as well as in the dog and cat, passive movements of the leg give rise to reflexes stimulant to respiration.

Discussion. The results of these experiments fully confirm Harrison's claims (6) (7) as to the existence of stimulant reflexes to respiration associated with movements of the limbs, but we are not prepared to accept his conclusion (6, p. 220) that: "The increase in ventilation produced by mild muscular movements is reflex in origin." We have no hesitation in affirming that these reflexes play a part, but from the data now available we are forced to conclude that the reflexes fall short in several important respects from the requirements of a complete explanation. One of these is the difference between various species of animals: we found that in the dog the reflexes aroused by passive movements stimulated the rate of breathing predominantly while in the cat the main effects

were on the depth and in man both rate and depth were stimulated. Another shortcoming has to do with time relationships: in the dog the reflex hyperpnea came on and ended abruptly, in the cat both onset and recovery were gradual, and in man the onset was usually abrupt but the recovery was frequently gradual. The findings in the cat come closest in both these respects to a satisfactory explanation for the hyperpnea of exercise, but in this animal the reflex component was relatively very weak and the stronger effects associated with active muscular contractions (ventral root stimulation) turned out to be due to direct stimulation of the center by a product of muscle metabolism (p. 539). Still another shortcoming of the reflex explanation is that it leaves no provision for adjustment of pulmonary ventilation to the work done, but only to the rate and extent to which the limbs are moved. It is common knowledge among

TABLE 2

Per cent change in respiration produced in man by passive movements of one leg

PATIENT NUMBER	BEFORE SPINAL ANESTHESIA		DURING SPINAL ANESTHESIA	
	Rate	M.V.	Rate	M.V.
1	+47	+51	+12.5	+15
	+22	+26	+6	+11
	+27	+28		
	+20	+39		
2	+18	+21	+5.5	+12.2
	+19	+29	+6.2	+15.8
3	+20	0	0	−7
	+20	+5.2	+6.2	+6.6
	+29	+43	+6.7	+2.5
	+20	+30		
	0	+22		
4	+13	+40	+4.5	0
	+13	+43		

cyclists that hyperpnea is much more closely related to the load than to the rate of pedaling, and a considerable mass of objective evidence to that effect is available from experiments on the bicycle ergometer (13, 16). The reflexes also have quantitative deficiencies, for the reflex effects thus far described are not nearly intense enough to justify the belief that they alone could cause the hyperpnea of muscular exercise, which is the strongest of which the organism is capable.

Final decision on these points must be deferred until other joints and other types of movement have been studied. At present we prefer to conclude that the reflexes constitute only one of several factors involved in this hyperpnea. This conclusion seems to us to be advisable in view not only of existing experimental evidence but also of the history of respiratory physiology, which has been characterized by a series of attempts, all of which have eventually turned

out to be mistaken, to explain too many observations on the basis of a single, simple theory. From the evidence now available we believe that "increased excitability" of the respiratory center during muscular exercise (i.e., hyperpnea without corresponding increase in cH or pCO_2 in the arterial blood) is probably due in part to excitatory afferent impulses from the limbs, in part to irradiation of excitation from cortico-spinal nerve fibers into the reticular formation of the medulla, and perhaps in part to afferent impulses from the lungs, aroused there by the changes in the pulmonary circulation associated with exercise. To attribute great importance to reflexes involving the pulmonary circulation is in line with the most recent trend of thought concerning the dyspnea of heart disease (2, 7), but evidence as to the applicability of this conception to normal subjects is lacking at present. Irradiation of excitations, originally suggested by Geppert and Zuntz (3), is the explanation favored by Krogh and his collaborators (12, 14); its part in the total respiratory response to exercise must necessarily be ascertained by exclusion when all other factors have been evaluated. As for reflexes from the limbs, it is possible (though in our opinion improbable) that the sum-total of all the afferent impulses aroused during muscular exercise will eventually afford an adequate explanation for the concomitant hyperpnea, particularly when there is also an increase in metabolic activity to prevent reduction in the stimulus level in the arterial blood during the hyperpnea. On the basis of *a priori* reasoning one would expect reflexes aroused in muscle chemoreceptors by accumulated products of muscle metabolism to be a much more important factor than reflexes from proprioceptors in the joints, and it is interesting to note that the first modern attempt at explaining the hyperpnea of exercise ascribed the major rôle to reflexes set up in the muscles by carbon dioxide (21, 22). Yet our results compel us to discard this attractive possibility and to conclude that, although respiration and circulation can be stimulated by reflexes aroused in the limbs by chemical substances, these phenomena are related to pain and not to a specific reflex system of physiological importance.

SUMMARY AND CONCLUSIONS

In human subjects exercise of one forearm and hand caused distinct hyperpnea, which was increased if the circulation was cut off. This potentiation by ischemia was probably due simply to pain. In analogous experiments on anesthetized dogs and cats, "exercise" of the hind-limbs (produced by stimulation of the ventral spinal roots) also caused hyperpnea, which in the dog was not influenced by ischemia but was abolished by transection of the spinal cord while in the cat it was reduced or abolished by ischemia, unaffected by cord transection. This hyperpnea therefore was due mainly to reflexes in the dog and in man, to direct central stimulation by chemical products of muscle contraction in the cat.

Various methods were used to detect a specific, chemosensitive reflex system in the limbs, without success. Passive movements however produced hyperpnea in dogs, cats and men, the effect being most marked in man and least marked in the cat. The reflex nature of this hyperpnea was proved by its absence after denervation or chordotomy in animals and by its diminution during spinal

anesthesia in man. By means of traction on muscles and tendons and of local anesthesia of the periarticular surfaces, the reflex was shown to arise largely or wholly in and around the knee joint, not in the muscles or tendons.

The possible significance of these findings to the respiratory response to muscular exercise is discussed and reasons are given for believing that while reflexes of this type unquestionably account for some of the hyperpnea, they probably cannot account for all of it.

REFERENCES

(1) Alam, M. and F. H. Smirk. J. Physiol. **89:** 372, 1937.
(2) Christie, R. V. Quart. J. Med. **7:** 421, 1938.
(3) Geppert, J. and N. Zuntz. Pflüger's Arch. **42:** 189, 1888.
(4) Gesell, R., C. A. Moyer and J. B. McKittrick. This Journal **136:** 486, 1942.
(5) Haldane, J. S. Respiration. Yale University Press, New Haven, 1921.
(6) Harrison, T. R. Failure of the circulation. Williams & Wilkins Co., Baltimore, 1939.
(7) Harrison, T. R., W. G. Harrison, Jr., J. A. Calhoun and J. P. Marsh. Arch. Int. Med. **50:** 690, 1932.
(8) Henderson, V. E. and H. V. Rice. J. Pharmacol. **66:** 336, 1939.
(9) Henderson, Y. Adventures in respiration. Williams & Wilkins Co., Baltimore, 1938.
(10) Heymans, C. and J. J. Bouckaert. Ergebn. d. Physiol. **41:** 28, 1939.
(11) Heymans, C. and L. Dautrebande. Arch. Internat. Pharmacodyn. **39:** 400, 1931.
(12) Krogh, A. Comparative physiology of respiratory mechanisms. University of Pennsylvania Press, Philadelphia, 1941.
(13) Krogh, A. and J. Lindhard. J. Physiol. **47:** 112, 1913.
(14) Krogh, A. and J. Lindhard. J. Physiol. **53:** 431, 1920.
(15) Moore, R. H., R. E. Moore and A. O. Singleton, Jr. This Journal **107:** 594, 1934.
(16) Nielsen, M. Skand. Arch. Physiol. **74:** supp. 10, 87, 1936.
(17) Ranson, S. W., W. F. Windle and L. R. Faubion. This Journal **64:** 320, 1923.
(18) Schmidt, C. F. Anesth. and Analg. **19:** 261, 1940.
(19) Schmidt, C. F. and J. H. Comroe, Jr. Physiol. Rev. **20:** 115, 1940.
(20) Sherrington, C. S. Mammalian physiology. Clarendon Press, Oxford, 1919, pp. 91–94.
(21) Vierordt. Wagner's Handwörterbuch d. Physiol., Braunschweig, 1844, vol. 2, p. 912.
(22) Volkmann. Müller's Arch., p. 342, 1841.
(23) Winder, C. V., T. Bernthal and W. F. Weeks. This Journal **124:** 238, 1938.
(24) Winterstein, H. Pflüger's Arch. **187:** 293, 1921.

THE EFFECT OF BILE IN THE INTESTINE ON THE SECRETION OF PANCREATIC JUICE

J. EARL THOMAS AND J. O. CRIDER

From the Department of Physiology of The Jefferson Medical College of Philadelphia

Received for publication November 2, 1942

The theory that bile in the intestine promotes the absorption of secretin and, therefore, increases the secretion of pancreatic juice was proposed by Mellanby (1926). In spite of the demonstration by Lueth and Ivy (1927) and by Dragstedt and Woodbury (1934) that as much pancreatic juice is secreted when bile is excluded from the intestine as when it is present, Mellanby's theory continues to receive prominent mention in modern textbooks of physiology (Cowgill, 1941; Best and Taylor, 1940; Evans, 1941). Although the evidence just cited eliminates the possibility that bile is necessary for the secretion of a normal amount of pancreatic juice during digestion, it leaves open the question of whether bile is capable of stimulating the pancreas in the absence of other stimuli. We have undertaken to investigate the action of bile alone or of bile mixed with various food products in the intestine on the secretion of pancreatic juice in fasting, unanesthetized animals.

METHODS. Three dogs were used for most of the work but some of the results have been repeated on two additional animals. The dogs were provided with gastric and duodenal fistulas with the duodenal fistula opposite the main pancreatic duct. Pancreatic juice was collected through a rubber funnel inserted by way of the duodenal fistula tube. In one dog the bile duct had been transplanted into the stomach. Since adequate drainage of the gastric contents to the outside was maintained in all of the dogs while observations were being made, in this animal only injected bile entered the intestine throughout the course of an experiment. This precaution did not, however, influence the results. Details of the methods have been described previously (Thomas and Crider, 1940; Thomas, 1941).

Two commercial preparations of desiccated ox bile were used as well as dog bile collected from the gallbladder of anesthetized animals or through the duodenal fistula from the animal under observation. The ox bile, with few exceptions, was dissolved in distilled water in whatever concentration was required to make the solution isotonic with the blood as indicated by determination of the freezing point. This concentration was 9 per cent for one preparation and 14 per cent for another. The pH of the bile preparations was generally measured and was, on occasion, adjusted to various predetermined levels in order to compare the effects of acid and alkaline bile.

The usual procedure, after preparing to record the flow of pancreatic juice with a drop recorder, was to wait until the flow had ceased or become constant and then inject 10 or 20 cc. of the bile preparation into the middle or lower duodenum or upper jejunum and record the result. In addition to experiments

devoted to the study of bile, it was used in a number of experiments designed for other purposes; as a result we have records of the action, or lack of action of bile, under a variety of experimental conditions.

RESULTS. *Attempts to stimulate pancreatic secretion by means of bile.* Only seven of a total of sixty-two injections of bile were followed by an increase in the rate of secretion of pancreatic juice apparently caused by the bile. A certain number of "positive" results are regularly obtained following the injection of any inert substance because of the appearance at unpredictable times of spontaneous secretion from the pancreas. Most of the results classed as positive could not be repeated in the same animal on the same day. In forty of the trials there was either no change or a decrease in the rate of secretion. In fifteen of the experiments the flow increased after bile was injected but either it had begun to increase before the injection was started or the increase was too small to be

TABLE 1

Effect on pancreatic secretion of injecting bile into the intestine

DOG NO.	NUMBER OF EXPERIMENTS		
	Increase	No effect or decrease	Doubtful
3-39	5	16	5
2-39	1	14	5
2-40	1	10	5
Total	7	40	15
pH RANGE			
2.75–6.9	1	9	7
7.0 –8.0	4	24	6
*Not known	2	7	2
Total	7	40	15

* Mostly dog's g.b. bile, therefore below pH 7.0.

considered significant (less than 1 drop per minute) or for some definite reason it was judged to be due to other causes. These results have been classified as doubtful. They should probably be considered negative.

We saw an unmistakable increase in the flow of pancreatic juice clearly due to the injection of bile on only two occasions. The increases occurred in experiments on the same animal (3-39) on two successive days. At the time his duodenal mucosa was intensely red and congested, suggesting duodenitis. After the mucosa returned to normal this animal again gave negative results. With this exception, the results were essentially the same in all three dogs.

In view of Mellanby's suggestion that the pH of the bile determines its effectiveness as a stimulus for the pancreas, we tried bile adjusted to various pH levels but observed no difference in the effects of acid as compared to alkaline bile. Results are summarized in table 1.

We also tried mixing the bile with various foodstuffs including dextrose, dextrin, peptones, fats and soap. These experiments gave the usual proportion of negative results with regard to the action of bile. In other experiments bile was administered when the stomach contained food or had recently been emptied through the gastric fistula. Generally under these circumstances the pancreas was secreting actively but the administration of bile failed to augment the secretion.

The inhibitory action of bile. Lueth and Ivy (1927) remarked that in some of their experiments administration of bile appeared to decrease the amount of pancreatic secretion produced in response to other agents. Dragstedt and Woodbury (1934) consistently obtained less pancreatic juice from their animals when bile was administered than when it was excluded from the intestine but

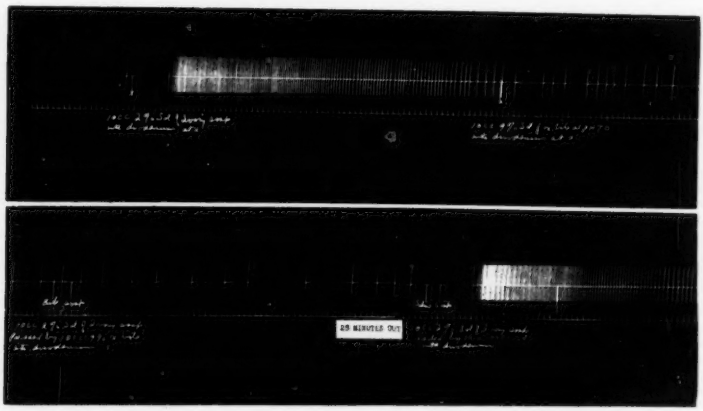


Fig. 1. Record made with a drop recorder showing the inhibitory effect of bile on the secretion of pancreatic juice due to soap (lower graph). The absence of any stimulating action of bile is also shown in the upper graph. The lower graph is a continuation of the upper one. Time is recorded in 30 second intervals.

they did not comment on the fact. In many of our experiments we have been impressed by our failure to obtain characteristic responses to other stimuli after administering bile. We, therefore, undertook a systematic study of the possible inhibitory action of bile on the response of the pancreas to other stimuli.

For this purpose two small injection tubes were passed into the intestine, one of which was between 13 and 17 inches longer than the other. Bile was injected through one (usually the longer) tube and either 5 per cent peptone solution, 2 per cent soap solution or N/20 HCl was injected through the other. With this arrangement the bile and the stimulating substance were placed in the intestine at different levels. This helped to prevent mixing and, in the case of HCl, neutralization.

In every instance, peptone, soap or HCl caused less secretion when given shortly after bile had been administered than it had previously produced when

given alone. Peptone or soap often failed to cause any secretion when bile was present and almost invariably failed if only moderate amounts of the stimulus (e.g., 10 cc.) were used. One such result is illustrated in figure 1. The results with HCl are presented in table 2.

DISCUSSION. In view of our failure to demonstrate any consistent increase in the flow of pancreatic juice following the administration of bile into the intestine of normal unanesthetized dogs, it is desirable to find the reason for Mellanby's positive results with anesthetized cats. One obvious explanation is that in a great majority of his experiments the pylorus was not tied and gastric juice, the secretion of which is stimulated by bile (J. Myer, A. C. Ivy and E. T. McEnery, 1942) was free to enter the intestine. In our experiments the gastric juice was drained to the outside through the gastric fistula. The long latent periods recorded by Mellanby and the fact that pancreatic secretion

TABLE 2

Amount of pancreatic juice secreted in response to 10 cc. N/20 HCl in the presence of bile compared to the normal response

Number of samples collected is given in parentheses beside the average volume in each instance

DOG NO.	DATE	WITHOUT BILE (AV. VOL. CC.)	WITH BILE (AV. VOL. CC.)	REMARKS
10–42	9/30/42	(3) 3.0	(4) 1.42	10 cc. N/10 HCl used as a stimulus
10–42	9/26/42	(4) 3.45	(4) 1.85	
11–42	10/12/42	(4) 2.52	(4) 0.47	10 cc. 1% $NaHCO_3$ injected into lower
11–42	9/24/42	(5) 3.12	(5) 0.55	tube in control experiments 10/12/42
2–40	4/ 7/41	(6) 10.6	(1) 5.2	Bile duct transplanted into stomach
2–40	4/ 9/41	(4) 6.66	(2) 3.4	
3–39	4/ 4/41	(1) 4.8	(1) 2.06	
3–39	4/ 8/41	(5) 6.66	(1) 4.7	

continued for hours suggest that his results were due to some such indirect mechanism. On the other hand he reported two experiments in which the pylorus and common bile duct were ligated. The results were less satisfactory in these experiments and because of the small number we cannot regard them as convincing. Nevertheless, there is reason to believe that bile may stimulate the pancreas under abnormal conditions such as frequently obtain in acute experiments (Thomas and Crider, 1941).

In contrast with the doubtful character of the evidence suggesting an excitatory effect of bile on pancreatic secretion, the evidence for an inhibitory effect on the response to various stimuli is clear and consistent. It is difficult to understand the usefulness of such an inhibitory mechanism unless it is involved in the regulation of pancreatic secretion. This is possible because most of the stimuli which increase the flow of pancreatic juice into the intestine also increase the flow of bile.

SUMMARY AND CONCLUSIONS

1. In normal unanesthetized dogs injection of ox bile or of dog bile into the intestine does not increase the rate of secretion of pancreatic juice.

2. The amount of pancreatic juice secreted in response to the presence of peptone, soap or HCl in the intestine is less when bile is also present than when it is absent from the intestine.

3. Mellanby's theory that bile is an important stimulus for pancreatic secretion is based on insufficient evidence and, in view of the contrary evidence, should be abandoned.

REFERENCES

BEST, C. H. AND N. B. TAYLOR. The physiological basis of medical practice. 2nd ed., p. 733, Baltimore, 1940.

COWGILL, G. R. McLeod's Physiology in modern medicine. 9th ed., p. 913, St. Louis, 1941.

DRAGSTEDT, L. R. AND R. A. WOODBURY. This Journal **107:** 584, 1934.

EVANS, C. L. Starling's Principles of human physiology. 8th ed., p. 913, Philadelphia, 1941.

LUETH, H. C. AND A. C. IVY. J.A.M.A. **89:** 1030, 1927.

MELLANBY, J. J. Physiol. **61:** 419, 1926.

MYER, J., A. C. IVY AND E. J. MCENERY. Arch. Int. Med. **34:** 129, 1924.

THOMAS, J. E. Proc. Soc. Exper. Biol. and Med. **46:** 260, 1941.

THOMAS, J. E. AND J. O. CRIDER. This Journal **131:** 349, 1940.
This Journal **133:** P469, 1941.

OBSERVATIONS CONCERNING THE ORIGIN OF RENAL LYMPH[1]

ALEX KAPLAN, MEYER FRIEDMAN AND H. E. KRUGER

From the Harold Brunn Institute for Cardiovascular Research, Mount Zion Hospital, San Francisco, California

Received for publication November 2, 1942

In a previous article (1) it was found that renal lymph contained more urea than the renal arterial and venous blood. Furthermore, it was found that the bulk of the supposedly large amount of fluid being reabsorbed by the tubules could not possibly re-enter the circulation via the renal lymphatics because of the minimal flow occurring in the latter vessels.

These findings posed two questions: 1, do the renal lymphatics drain only the larger collecting ducts of the kidney, thus accounting for the high urea content of its lymph, and 2, is renal lymph derived from the tubular reabsorbed fluid, the blood plasma or from both types of fluid.

In the present study, an attempt was made to answer the first question by a determination and comparison of the glucose content of renal and cervical lymph samples. For if renal lymph were derived from the fluid in the larger renal collecting ducts (which supposedly contains a high urea and a negligible glucose content), it would be expected that renal lymph contained a much lower glucose concentration than cervical lymph, the latter type of lymph having as much glucose as blood plasma (2).

In order to solve the second question, the inulin content of renal lymph (during the intravenous infusion of inulin) was determined and compared with that of cervical lymph for the latter is derived from blood plasma. If renal lymph is derived from, and is in diffusion equilibrium with tubular reabsorbed fluid alone, it will have little or no inulin since tubular reabsorbed fluid assumedly does not contain inulin (3); if its inulin content is equal to that of cervical lymph, it is derived from and is in diffusion equilibrium ostensibly with renal blood plasma alone; and finally, if renal lymph is in diffusion equilibrium with both the renal blood plasma and tubular reabsorbed fluid, it will have some inulin but certainly, considerably less than that in cervical lymph.

METHODS. For the determination of the glucose in renal lymph, the hilar and capsular lymphatics of the dog's kidney were cannulated according to a method previously described (1). Approximately 0.025 cc. samples of renal lymph were deproteinized according to the method of Giragossintz, Davidson and Kirk (4). After subsequent centrifugation, 0.05 cc. of the supernatant fluid was oxidized with potassium ferricyanide reagent and titrated with ceric sulfate according to the method of Giragossintz and his associates. Arterial blood (5.0 cc.) was collected at the beginning and end of the lymph cannulation. After centrifugation under oil, the plasma was tested for its glucose content exactly as described above for the glucose determination of lymph. Two renal

[1] Aided by a grant from the Dazian Foundation for Medical Research.

lymph samples were obtained from the capsular lymphatics and the remaining seven samples were obtained from the hilar lymphatics of the kidney. For control purposes, seven samples of cervical lymph were obtained at the same time that renal lymph samples were obtained, and they were analyzed for glucose exactly as described above.

For the determination of the inulin content of renal lymph, eight large dogs (18–22 kgm.) were given an infusion of 250 cc. of normal saline solution containing 20 grams of inulin beginning 30 minutes prior to lymph duct cannulation and continuing during the collection. After the lymph duct was cannulated, arterial blood (femoral) was obtained and urine was collected from a catheter inserted into the ureter of the kidney. In three of these eight dogs, lymph samples also were obtained from the left cervical lymphatic vessel at the same time that renal lymph was being collected. Two more cervical lymph samples were

TABLE 1

The glucose content of renal and cervical lymph

EXP. NO.	BLOOD PLASMA GLUCOSE (MGM./100 CC.)	RENAL LYMPH GLUCOSE (MGM./100 CC.)	CERVICAL LYMPH GLUCOSE (MGM./100 CC.)
12	135.0	115.0	
13	107.0		98.0
14	70.0	70.0	101.0
16	106.0	86.0	97.0
17	102.0	92.0	84.0
18	99.0	108.0*	107.0
19	113.0	98.0	125.0
20	99.0	90.0	91.0
21	101.0	83.0	112.0
Average	103.5	92.7	101.9

* Capsular lymph.

obtained from two normal dogs, receiving an inulin infusion similar to that given to the eight dogs above. After 20 minutes of renal lymph collection, a second arterial blood sample was taken and the urine collection was stopped. The inulin content of the lymph samples (renal and cervical), the two blood samples, and the urine sample was determined according to the method of Alving, Rubin and Miller (5).

RESULTS. A. *The glucose content of renal and cervical lymph.* As can be seen in table 1, the average concentration of glucose in eight renal lymph samples was 92.7 mgm. per 100 cc. and 101.9 mgm. in the eight cervical lymph samples. This high glucose concentration in renal lymph strongly suggests that renal lymph could not be derived exclusively from the relatively sugar-free fluid contained in the larger collecting ducts of the kidney. The close similarity, however, between the glucose concentrations of renal and cervical lymph samples does not of itself indicate that renal lymph is derived exclusively from renal blood plasma, for it may be derived partially from tubular reabsorbed fluid and

still have a glucose content as high as cervical lymph, since tubular reabsorbed fluid also is supposedly high in glucose.

B. *The inulin content of renal and cervical lymph.* The average concentration of inulin in renal lymph samples taken from eight dogs receiving an intravenous infusion of inulin (and having an average blood plasma concentration of 121.2 mgm. per 100 cc.) was found to be 82.5 mgm. per 100 cc. (see table 2). It was found also that cervical lymph, collected under the same experimental conditions, contained an average inulin concentration of 138.8 mgm. as compared to an average blood plasma concentration of 147.6 mgm. Thus, unlike the similarity in the glucose values of renal and cervical lymph samples, a marked difference in the inulin content of these two types of lymph was found. For whereas cervical lymph had an inulin content 94 per cent of that in blood plasma, renal lymph had an inulin content but 68.0 per cent of that found in blood

TABLE 2

The inulin content of renal and cervical lymph

EXP. NO.	BLOOD PLASMA INULIN (MGM./100 CC.)	RENAL LYMPH INULIN (MGM./100 CC.)	CERVICAL LYMPH INULIN (MGM./100 CC.)	INULIN CLEARANCE (CC./MIN.)	URINE VOLUME (CC./MIN.)
II	82.7	43.8*		57.4	0.84
IV	56.8	10.0*		61.8	0.67
V	194.2	142.0*		49.0	1.87
IX	108.0	81.8		36.5	1.63
X	65.9	35.0		127.0	2.20
XI	136.0		142.8		
XII	140.0		144.8		
XIII	16.0	10.8	20.0	36.9	0.98
XIV	84.0	70.0	99.5	38.3	0.97
XV	362.0	266.4	287.0	53.6	0.80
Average.....	147.6†	82.5	138.8	57.6	1.25
	121.2‡				

* Capsular lymph.

† Average blood level of inulin during collection of cervical lymph.

‡ Average blood level of inulin during collection of renal lymph.

plasma. It is of interest that the average inulin clearance of the single kidney in these experiments was 57.6 cc. per minute. This large clearance indicates that these kidneys were functioning in good order despite the cannulation procedure effected upon one of their lymphatic vessels.

DISCUSSION. The preceding observations make it clear that renal lymph is not derived exclusively from the relatively sugar-free fluid contained in the larger collecting ducts of the kidney. For if it were, the glucose content of renal lymph would be nil or very low whereas it was found that its content of this substance was practically as high as that of cervical lymph. Thus, the high urea content of renal lymph (1) cannot be explained by the assumption that renal lymph is derived chiefly from the fluid contained in the larger collecting ducts which is high in urea.

The above experiments also appear to indicate that the composition of renal lymph is determined by the character of both tubular reabsorbed fluid and the renal blood plasma. If it were derived exclusively from the renal tubular reabsorbed fluid, its inulin content would be practically nil, and if it were exclusively derived from the renal blood plasma, its inulin content would be equal to that of cervical lymph. In view of the fact that renal lymph does contain considerable inulin but at a considerably lower concentration than that found in cervical lymph, it seems reasonable to assume that it is derived from both fluid elements present in the kidney. As mentioned above, the glucose content of renal lymph, unlike the inulin content, was approximately the same as that of cervical lymph. However, this is to be expected, regardless of the source of renal lymph, since tubular reabsorbed fluid supposedly contains as much glucose as the renal blood plasma.

CONCLUSIONS

1. The glucose and inulin contents of renal and cervical lymph were determined and compared.

2. Renal lymph is not derived exclusively from the larger collecting ducts of the kidney, despite the high concentration of urea in renal lymph.

3. Renal lymph is derived apparently from both the renal blood plasma and the tubular reabsorbed fluid.

The authors wish to express their thanks to E. Lindner and Ralph Levy for their technical assistance.

REFERENCES

(1) Sugarman, J., M. Friedman, E. Barrett and T. Addis. This Journal. To be published.

(2) Drinker, C. K. and J. M. Yoffee. Lymphatics, lymph and lymphoid tissue. Harvard Univ. Press, Cambridge, 1941.

(3) Smith, H. W. The physiology of the kidney. Oxford Univ. Press, New York, 1937.

(4) Giragossintz, G., C. Davidson and P. L. Kirk. Mikrochemie **21:** 21, 1936.

(5) Alving, A. S., J. Rubin and B. F. Miller. J. Biol. Chem. **127:** 609, 1939.

THE ABSENCE OF RENNIN FROM ADULT HUMAN GASTRIC JUICE[1]

LOUIS B. DOTTI AND ISRAEL S. KLEINER

From the Department of Physiology and Biochemistry, New York Medical College, Flower and Fifth Avenue Hospitals

Received for publication November 9, 1942

It is now generally accepted that pepsin and rennin are distinct and different enzymes (1, 2). However, the impression seems to be general that they are both present in human gastric juice. Most text-books make such statements and, indeed, some investigators have made similar assumptions. For example, Helmer, Fouts and Zerfas (3, 4) determined the milk-clotting power of human gastric juice and designated as "rennin" the value thus obtained, while estimating "pepsin" by another procedure. Since pepsin also clots milk, obviously their "rennin" values are really either pepsin plus rennin or pepsin alone. There is no evidence to support the assumption that rennin is present in human gastric juice.

Holter and Anderson (2, 5) studied the pepsin/rennin quotient of the gastric fluids of human subjects as well as of other species. They found that this quotient is fairly constant in the human and can not be changed by chemical influences. Their studies led them to the conclusion that rennin is produced only by the calf, and, presumably, by other ruminants.

Although Holter and Anderson's work is quite convincing, it is, nevertheless, indirect. It seemed to us that a more direct approach to the problem might be made. Tauber and Kleiner (6) showed that pepsin solutions rather rapidly digest rennin. This seemed to afford a means of detecting rennin in gastric juice. If the clotting power of gastric juice, determined soon after it is collected, suffers considerable reduction on incubation, it may be considered evidence of the digestion of the rennin by the pepsin-HCl. On the other hand, if no diminution of power takes place in gastric juice containing active pepsin it would show that no rennin is present.

The technique used to measure the clotting-power was the Barowsky-Tauber-Kleiner clinical method of estimating pepsin (7). Its basis is the determination of the smallest quantity of diluted gastric juice that will just clot 10 cc. of milk, buffered to pH 5.0 in ten minutes.

Gastric fluid was obtained from adult subjects following a "test meal" of 150 cc. of 7 per cent ethyl alcohol or a histamine injection. If the fluid was not acid, it was acidified to congo red. The clotting power was immediately determined. The sample was then incubated at 38°C. for 24 hours, after which the clotting power was again measured.

The table (table 1) gives the results of 15 experiments. In one case (no. 6) there is a slight increase in activity, possibly indicating a completed conversion

[1] A preliminary report of this work was presented before the American Physiological Society at Boston, Mass., April 3, 1942; Federation Proceedings **1:** 21, 1942.

of pepsinogen to pepsin. In two experiments (nos. 11 and 13) a slight decrease might be interpreted as due to a trace of rennin. However, they represent a difference of 0.1 and 0.2 cc. of gastric juice added, respectively.[2] We are inclined to regard them as errors due to the method. All the other twelve experiments show no change in clotting power.

To check the remote possibility that the entire clotting power was due to rennin rather than pepsin, a 1:1 mixture of the gastric fluid and pepsin solution (containing 833 units/cc.) was incubated for 24 hours. This was done in five experiments (nos. 6–10). The differences between the amounts found and those calculated, assuming no rennin was present, are inconsequential.

TABLE 1

SAMPLE NUMBER	GASTRIC FLUID AS COLLECTED (PEPTIC UNITS PER CC.)	GASTRIC FLUID AFTER INCUBATION AT 38°C. FOR 24 HOURS (PEPTIC UNITS PER CC.)	10 CC. OF GASTRIC FLUID PLUS 10 CC. OF RENNIN SOLUTION (833 UNITS/CC.) AFTER INCUBATION AT 38°C. FOR 24 HOURS		10 CC. OF GASTRIC FLUID PLUS 10 CC. OF PEPSIN SOLUTION (833 UNITS/CC.) AFTER INCUBATION AT 38°C. FOR 24 HOURS	
			Peptic units found	Peptic units calculated assuming all rennin destroyed	Peptic units found	Peptic units calculated assuming no rennin present
1	500	500				
2	667	667				
3	625	625				
4	625	625	312	312		
5	357	357	180	180		
6	714	1250	625	625	833	1041
7	1250	1250	625	625	1000	1041
8	5000	5000	2500	2500	3333	2917
9	1667	1667	834	834	1250	1250
10	2500	2500	1250	1250	1666	1666
11	1000	833	417	417		
12	833	833	500	417		
13	625	500	417	313		
14	714	714	417	357		
15	313	313	179	157		

In order to be sure that the conditions were suitable for digestion of rennin, a 1:1 mixture of gastric fluid and rennin solution (833 units/cc.) was likewise incubated. In nearly all the 12 experiments in which this was tried all the rennin was digested in 24 hours.

We have other records of the same general tenor. In some instances human gastric fluid remained at refrigerator temperature for days and even weeks with no change in clotting power. Corroboration of these results has been obtained

[2] The empirical formula for calculating the unitage is Units/cc. = (1/volume of diluted gastric fluid × 10) × degree of dilution. The dilutions are (1) undiluted, (2) 1:25 or 1:50 and the volumes used are 0.1 to 0.9 cc. in 0.1 cc. increments. Thus, a change of 0.1 cc. gives a fairly large variation in the total number of units found. A clinical unit is extremely small being equivalent to 0.04 gamma pepsin nitrogen. This value was determined by using a solution of crystalline pepsin for which we wish to thank Dr. J. H. Northrop.

with samples from normal and pathological hospital cases (8). The fact that all clotting power of human gastric juice is due to pepsin alone would explain the fact that pepsin and "rennin" seem to run parallel in normal and pathological cases, according to Helmer, Fouts and Zerfas' observations.

SUMMARY

By using the phenomenon of the digestibility of rennin by pepsin as a basis for the detection of rennin, it was found that no rennin was present in the gastric juice of human adults.

REFERENCES

(1) TAUBER, H. AND I. S. KLEINER. J. Biol. Chem. **96:** 745, 1932.
(2) HOLTER, H. AND B. ANDERSON. Biochem. Ztschr. **269:** 285, 1934.
(3) HELMER, O. M., P. J. FOUTS AND L. G. ZERFAS. J. Clin. Investigation **11:** 1129, 1932.
(4) HELMER, O. M., P. J. FOUTS AND L. G. ZERFAS. Am. J. Digest. Dis. **1:** 120, 1934.
(5) ANDERSON, B. Biochem. Ztschr. **262:** 99, 1933.
(6) TAUBER, H. AND I. S. KLEINER. J. Biol. Chem. **104:** 259, 1934.
(7) BAROWSKY, H., H. TAUBER AND I. S. KLEINER. Am. J. Digest. Dis. **4:** 229, 1937.
(8) Personal communication from Dr. Louis Malinash; to be published elsewhere.

HISTOLOGICAL STUDIES OF THE PANCREAS AND ASSOCIATED TISSUES OF WILD AND EXPERIMENTALLY FED YOUNG CHINOOK SALMON

LAUREN R. DONALDSON

From the School of Fisheries, University of Washington

Received for publication November 14, 1942

The general results of two feeding experiments using chinook salmon fingerlings (*Oncorhynchus tschawytscha*) as test animals were described by Donaldson and Foster (1939) and Norris and Donaldson (1940). The pancreatic-intestinal region of representative fish of each lot in the experiments mentioned was preserved at the end of the experiment and subjected to a histological study. The results of this study indicate very pronounced changes may take place in the pancreas of chinook salmon fingerlings as a result of various diets.

Gaschott (1931), Hayford and Davis (1936), and Hewitt (1937a and 1937b) described diseased conditions in trout livers produced by faulty diet. Hess (1935) found that profound changes occurred in the pancreas of fish under different dietary and environmental conditions.

The studies of Hess, of Dragstedt et al. (1936) and of Van Prohaska et al. (1936) on liver changes in depancreatized dogs suggested that the pancreatic changes might be closely linked with fatty degeneration of the liver and might, in fact, precede the liver changes. The studies on chinook salmon carried on to date seem to indicate that such is the case.

Discussion of the histological condition of wild and experimentally fed fish. A study of normal pyloric ceca-pancreatic tissue preceded the study of the material from the fish of the experimental lots. Chinook salmon were obtained from wild stocks in areas where it is almost certain they had not received other than natural food substances. Representative tissue from a wild fish, with standard length of 53 mm., is presented in figure 1.

The pancreatic tissue of these wild fish has very dense acinar tissue, the cells of which are distinct. Fat is interspersed among the ceca, or in some cases infiltrates into the acinar tissues of the pancreas. The tissues from fish with lengths of 49, 53, 87 and 102 mm. show very definite fat deposits in the pancreatic tissue. The tissues from a 32-mm. fish, a fish just beginning to feed, show very little fat infiltration in the pancreatic tissues but a generous amount of fat tissue is located between the loops of the intestine. A single fish, one 69 mm. in length, failed to reveal fat tissue deposited either among the ceca or in the pancreatic tissue.

The tissues from the region of the pyloric ceca and intestine of the fish studied are similar to the tissues described by Greene (1914, 1915) from young chinook salmon, and Barr (1935) from the adults.

The cross sections of the intestine, pyloric ceca, and pancreas of the fish fed experimentally show some with tissues very similar to those of the wild fish, and others with marked changes in the tissues.

The cross sections of the liver-fed fish, lot 1, reveal tissues very similar to those figured and described by Hess (1935). Considerable fatty infiltration of the pancreas has taken place. The acinar cells that remain, however, appear to be normal. Islets of Langerhans occur scattered throughout the tissue. The infiltration of the fat in this case was not reflected in any adverse condition noted externally in the fish. The rate of growth and low mortality at the termination of the experiment lead one to conclude the fat infiltration in this case was not excessive in amount.

Cross sections of pancreatic tissue in the fish fed a diet of salmon viscera alone show very marked differences from those of fish that were fed an exclusive beef liver diet. The amount of fatty infiltration was much less in the pancreas of the fish fed salmon viscera. The acinar tissue has a very indistinct appearance, with some cell material evidently breaking from the cells and lodging in the spaces between the pyloric ceca and the pancreatic tissue. This condition is indicative of the initial stages of the breakdown of the tissue. The mortality rates and rate of growth of the fish in this experimental lot were such that one might assume the condition was not, as yet, extremely injurious to the fish.

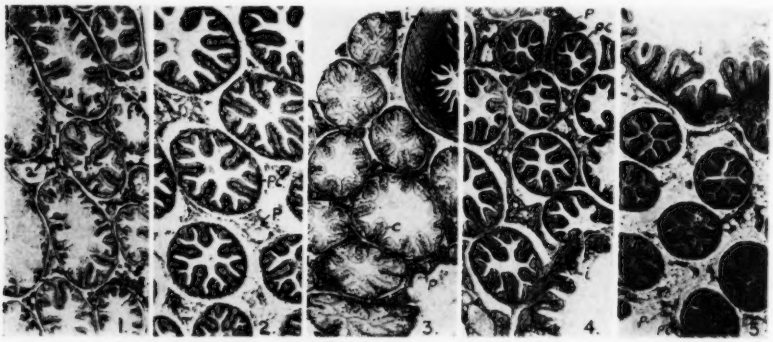
The fish in lot 3, fed a diet of 40 per cent beef liver and 60 per cent seal meal, present a condition slightly more aggravated than the fish in lot 2. The pancreatic tissue in these fish appeared to be in the initial stages of degeneration, with some breakdown in the cells. The mortality increased during the last two weeks of the experiment,—a further indication of an incipient dietary deficiency.

The fish fed diet 5, composed of 20 per cent beef liver, 20 per cent salmon viscera and 60 per cent seal meal show, both macroscopically and microscopically, all the symptoms of the diseased condition usually referred to as fatty degeneration of the liver. In addition these fish contained pancreatic tissue in the advanced stage of degeneration (fig. 2). The actual pancreatic tissue had largely disappeared leaving only cell fragments, connective tissue, and fat cells between the pyloric ceca. A marked increase in the mortality rate further indicated that the diet was inadequate for the young fish.

The fish that were fed a diet of one-third beef liver, one-third salmon viscera, and one-third seal meal developed pancreatic tissues that were apparently normal. The tissues of these fish, with an average length of 63.7 mm., are very similar to those of wild fish of about the same length.

Fish that received 20 per cent ground beef pancreas, 20 per cent beef liver, and 60 per cent seal meal as their diet, lot 7, developed pancreatic tissue which appeared very similar to that produced by fish fed a diet of 100 per cent beef liver. The large amount of fatty tissue in the pancreas of the fish in this experimental lot may have been produced by the large amounts of beef fat that were mixed with the beef pancreatic tissues. The very remarkable differences in the tissues of the fish fed diets 3, 5 and 7, demonstrate clearly a pronounced effect produced by dietary changes. The replacing of 20 per cent of the beef liver of diet 3 with the 20 per cent beef pancreas in diet 7, resulted in a great increase in the fatty tissue in the fish pancreas. The acinar tissue of the fish

fed diet 7 appeared to be distinct and normal. The differences in the pancreatic tissue of the fish fed diet 7 with 20 per cent beef pancreas, as compared with the fish that received diet 5, which contained 20 per cent salmon viscera, would lead one to conclude that the addition of beef pancreas to the diet had a very



The photomicrographs of the cross sections through the region of the pyloric ceca, pancreas, and intestine were made with uniform magnification (× 30). The variation in size of the pyloric ceca is due largely to the variation in size of the fish from which the materials were removed.

Abbreviations: *a*—acinar tissue of the pancreas; *c*—columnar epithelium; *f*—fat tissue; *i*—intestine; *p*—pancreas; *p.c.*—pyloric ceca.

Fig. 1. Photomicrograph of the cross section of the pancreas, pyloric ceca, and intestine of a 53 mm.-chinook salmon fingerling captured in the San Joaquin River, California.

Fig. 2. Photomicrograph of the cross section of the pancreas and pyloric ceca of a chinook salmon fingerling from a group averaging 64.6 mm. in length that had been fed diet 5 (20 per cent beef liver, 20 per cent salmon viscera, 60 per cent seal meal) for twenty weeks. The pancreatic tissue shows almost complete degeneration.

Fig. 3. Photomicrograph of a cross section of the pancreas, pyloric ceca, and intestine of a chinook salmon fingerling from a group averaging 42.6 mm. in length that had been fed diet 11 (basic diet + 20 parts of salmon oil, 2 parts of cholesterol) for sixteen weeks. The pancreatic tissue shows some degeneration. The columnar epithelium of the pyloric ceca is greatly thickened and vacuolated.

Fig. 4. Photomicrograph of a cross section of the pancreas, pyloric ceca, and intestine of a chinook salmon fingerling from a group averaging 49.3 mm. in length that had been fed diet 12 (basic diet + 20 parts of starch, pancreas extract) for sixteen weeks. The pancreatic tissues are apparently normal.

Fig. 5. Photomicrograph of a cross section of the pancreas, pyloric ceca, and intestine of a chinook salmon fingerling from a group averaging 50.2 mm. in length that had been fed diet 13 (basic diet + 20 parts of salmon oil, 2 parts of cholesterol, pancreas extract) for sixteen weeks. The pancreatic tissue is almost completely disintegrated.

beneficial effect, while the salmon viscera in a similar diet had a very injurious effect on the pancreatic tissues of the young chinook salmon.

Twenty per cent apple flour combined with 40 per cent seal meal and 40 per cent beef liver, and fed to fish in lot 8, resulted in pancreatic tissue very similar to that of fish fed diet 3. The supplementing of part of the seal meal with 20 per cent apple flour did not produce a noticeable difference in the pancreatic tissues.

Pancreatic changes in fish fed synthetic diets. The growth and mortality rates of the fish fed on synthetic diets (nos. 9–14) were discussed in a previous report (Norris and Donaldson, 1940).

The fish fed diet 9, in which 80 parts of the basic diet[1] were supplemented by the addition of 20 parts of starch, showed very little structural modification of the tissues.

Pancreatic tissues of the fish fed diet 10, in which 80 parts of the basic diet were supplemented with 20 parts of salmon oil, show very pronounced degeneration.

The tissues of the fish fed diet 11, in which 80 parts of the basic diet were supplemented with 20 parts of salmon oil and 2 parts of cholesterol (fig. 3), show most extraordinary modification. The cells of the columnar epithelium of the pyloric ceca and parts of the intestine are greatly enlarged. The condition of these cells is similar, although much more pronounced than that of tissues from the same region of young chinook salmon, figured by Greene (1915), that were fed olive oil by rectal injection.

The addition of pancreatic extract and 20 parts of starch to the basic diet (12) produced pancreatic tissues that appeared to be very nearly normal. The photomicrograph of tissues from representative fish from this lot (fig. 4), of an average length of 49.3 mm. is very similar to those of wild fish of 49 mm. length. The differences in the tissues of the fish fed diet 12 and those fed diet 9 may be attributed to the adding of the pancreatic extract.

The beneficial effect of feeding the pancreatic extracts in this case is similar to the results obtained by Van Prohaska et al. (1936) and Dragstedt et al. (1936), who found that the "fat-free alcohol extract" of beef pancreas contained an active principle that was effective in preventing fatty liver in depancreatized dogs. To this active substance they gave the name "lipocaic" and described it as a fat metabolizing hormone.

Figure 5 represents a photomicrograph of the cross section of fish fed diet 13. This diet consisted of the basic diet supplemented with 20 parts of salmon oil, 2 parts of cholesterol, and pancreatic extract. The pancreatic tissues show almost complete disintegration while the columnar epithelium cells of the pyloric ceca appear normal. The differences between the tissues photographed in figure 5 and those in figure 3 can be said to be due to the addition of pancreatic extract in the former case. The differences between the fish tissues reproduced in figures 5 and 4 can be attributed to the addition of salmon oil and cholesterol in the diet of the former, to replace the starch in the latter diet.

The addition of choline-hydrochloride to diet 14, which in addition to the basic diet contained 20 parts of salmon oil and 2 parts of cholesterol, seemed to have some beneficial effect in preventing abnormal changes in the tissues. This result parallels the findings of Halliday (1938) who found that the addition of choline to the diets of rats suffering from fatty liver exerted a remedial effect.

It might be concluded that the addition of 20 parts of salmon oil, or salmon oil and 2 parts of cholesterol, to the basic diet produced destructive changes in

[1] The basic diet used was composed of the following parts: meat 55, salt 5, yeast 8, cod liver oil 2, gelatin 10, and liver extract.

the pancreatic tissue. The addition of pancreatic extract of choline to the diets exerted a beneficial effect in preventing these changes.

SUMMARY AND CONCLUSIONS

Representative fish from experimental lots of fish fed various diets were preserved, and the pyloric ceca-pancreatic region studied by cross sectioning the area and comparing it with tissues from the same region from wild fish.

These studies indicate that profound changes occur in the pancreatic tissue of fish as a result of the diet.

The fish fed a diet of 100 per cent beef liver had excessive fat deposited in the pancreatic tissues.

Diets of 20 per cent salmon viscera, 20 per cent beef liver, and 60 per cent seal meal induced degeneration of the pancreatic tissue, while fish fed a diet of $\frac{1}{3}$ salmon viscera, $\frac{1}{3}$ beef liver, and $\frac{1}{3}$ seal meal had normal appearing pancreatic tissue.

Fresh beef pancreas in the diet added to the fat content of the fish pancreas, but in other respects the fish pancreatic tissues appeared normal.

The addition of salmon oil, or salmon oil and cholesterol to synthetic diets produced destructive changes in the fish pancreatic tissues. The addition of pancreatic extract or choline to the synthetic diets produced beneficial effects in preventing the destructive changes.

REFERENCES

(1) Barr, C. H. Thesis, Univ. of Oregon, 1935.
(2) Donaldson, L. R. and R. F. Foster. Prog. Fish-Cult. **44:** 10, 1939.
(3) Dragstedt, L. R., J. Van Prohaska and H. P. Harms. This Journal **117:** 175, 1936.
(4) Gaschott, O. Salmon and Trout Mag. **64:** 273, 1931.
(5) Greene, C. W. Bull. Bur. Fish. **32:** 73, 1914.
(6) Greene, C. W. Bull. Bur. Fish. **33:** 149, 1915.
(7) Halliday, N. J. Nutrition **16:** 285, 1938.
(8) Hayford, C. O. and H. S. Davis. Prog. Fish-Cult. **17:** 7, 1936.
(9) Hess, W. N. J. Exper. Zool. **70:** 187, 1935.
(10) Hewitt, E. R. Trans. Am. Fish. Soc. **66:** 291, 1937.
(11) Hewitt, E. R. Prog. Fish-Cult. **27:** 11, 1937.
(12) Norris, E. R. and L. R. Donaldson. This Journal **129:** 214, 1940.
(13) Van Prohaska, J., L. R. Dragstedt and H. P. Harms. This Journal **117:** 166, 1936.